No. 91 VOLUME XXII NOVEMBER 1961

American Journal of

Veterinary RESEARCH

In this issue . . .

- Oxalate Formation of Moldy Feedstuffs in Livestock Toxic Disease 961
- Urinary Biocolloids of Sheep 1000
- Microbiologic Survey of Pneumonic Swine Lungs . . 1020
- Contrast Encephalography and Ventriculography in Dogs 1041

CONTENTS ARE ON PAGES 953, 958, 959

SURITAL®
SODIUM

(thiamylal sodium, Parke-Davis)

ultrashort-acting
intravenous
anesthetic

smooth
anesthesia

for surgical, orthopedic, and dental procedures

- . . . SUITABLE FOR BOTH LARGE AND SMALL ANIMALS OF ANY AGE GROUP
- . . . INDUCTION: RAPID AND SMOOTH
- . . . RECOVERY: GENERALLY RAPID AND UNCOMPLICATED

Information regarding details of administration, precautions, and dosage available on request.

PARKE-DAVIS PARKE, DAVIS & COMPANY, Detroit 32, Michigan

#249-1061

American Journal of Veterinary Research

Volume XXII November, 1961 Number 91

Contents

The articles in the Research Journal are grouped, loosely, by animal species, followed by the more general articles. The articles listed on the cover are those that appear at the beginning of the various divisions or are particularly representative of a number of similar articles in a division.

Oxalate Formation in Moldy Feedstuffs as a Possible Factor in Livestock Toxic Disease 961
B. J. Wilson and C. H. Wilson

Studies on Bovine Staphylococcic Mastitis. II. Characterization of Isolates 970
L. W. Fleming and I. M. Paton

Quantitative Coagulase Activity and Phage Patterns of Strains of *Staphylococcus aureus* Isolated from Cows in Michigan 975
J. J. Solomon, C. L. San Clemente, and A. R. Drury

The Comparative Susceptibility of Calves and Adult Cattle to Bovine Anaplasmosis 982
T. O. Roby, D. W. Gates, and L. O. Mott

Polysaccharide and Protein Relationships of Normal Bovine Serum 987
G. T. Dimopoullos

A Technique for the Surgical Removal of the Forestomachs of Calves 990
F. Sauer and G. J. Brisson

Serologic Studies Suggestive of *Leptospira hardjo* in a Beef Herd 995
M. A. Ramirez, R. C. Allen, W. R. Van Dresser, and D. F. Watson

Ruminant Urolithiasis. IV. Ultrafiltration, Electrophoretic, and Chromatographic Studies on Urinary Biocolloids of Sheep 1000
C. E. Cornelius, J. A. Bishop, L. R. Berger, and J. Pangborn

The Creatinine Coefficient of Normal Sheep 1012
M. Z. Barakat and A. Abdalla

Factors Influencing the Incidence of Chronic Pneumonia in Lambs 1015
A. Lagacé, W. D. Pounden, D. S. Bell, and K. D. Weide

Microbiologic Survey of Pneumonic and Normal Swine Lungs 1020
C. L'Ecuyer, W. P. Switzer, and E. D. Roberts

Serum Transaminase and Aldolase During Migration of Larval *Ascaris suum* in Swine 1026
M. F. Andrews, P. K. McIlwain, and D. F. Eveleth

(Continued on page 958)

PRINTED IN U.S.A.

Council on Research of the AVMA

CARL A. BRANDLY, Urbana, Ill.
GEORGE T. EDDS, Fort Dodge, Iowa
WILLIAM A. HAGAN, Ames, Iowa
RUE JENSEN, Fort Collins, Colo.
T. CARL JONES, Boston, Mass.
R. L. KITCHELL, St. Paul, Minn.
JOHN T. MCGRATH, Drexel Hill, Pa.
GEORGE C. POPPENSIEK, Ithaca, N.Y.
C. ROGER SMITH, Columbus, Ohio
RICHARD D. TURK, College Station, Texas

Committee on Journal of the Council on Research

CARL A. BRANDLY, WILLIAM A. HAGAN

Reviewers of Research Manuscripts

JOSEPH O. ALBERTS, Urbana, Ill.
JAMES ARCHIBALD, Guelph, Ont.
WILFORD S. BAILEY, Auburn, Ala.
CHARLES N. BARRON, Philadelphia, Pa.
WILLIAM E. BROCK, Stillwater, Okla.
M. LOIS CALHOUN, East Lansing, Mich.
CLARENCE R. COLE, Columbus, Ohio
CHARLES H. CUNNINGHAM, East Lansing, Mich.
DAVID K. DETWEILER, Philadelphia, Pa.
ROBERT W. DOUGHERTY, Ithaca, N.Y.
HOWARD W. DUNNE, University Park, Pa.
LLOYD C. FERGUSON, East Lansing, Mich.
JEAN C. FLINT, Fort Collins, Colo.
JOHN R. GORHAM, Pullman, Wash.
DONALD P. GUSTAFSON, Lafayette, Ind.
JAMES A. HENDERSON, Guelph, Canada
WILLIAM KAPLAN, Chamblee, Ga.
ALFRED G. KARLSON, Rochester, Minn.
B. W. KINGREY, Ames, Iowa
WENDELL KRULL, Stillwater, Okla.
ROGER P. LINK, Urbana, Ill.
HADLEIGH MARSH, Bozeman, Mont.
ROBERT R. MARSHAK, Philadelphia, Pa.
ERSKINE V. MORSE, Lafayette, Ind.
CARL OLSON, Madison, Wis.
RICHARD L. OTT, Pullman, Wash.
R. ALLEN PACKER, Ames, Iowa
WILLIAM R. PRITCHARD, Gainesville, Fla.
STEPHEN J. ROBERTS, Ithaca, N.Y.
ARNOLD S. ROSENWALD, Davis, Calif.
JOHN H. RUST, Chicago, Ill.
H. R. SEIBOLD, Plum Island, N.Y.
ALVIN F. SELLERS, Ithaca, N.Y.
HERBERT G. STOENNER, Hamilton, Mont.
MELVIN J. SWENSON, Ames, Iowa
WALTER G. VENZKE, Columbus, Ohio
CHARLES K. WHITEHAIR, East Lansing, Mich.

EDITORIAL STAFF: DR. D. A. PRICE, *Editor-in-Chief*; MRS. EVA G. BAILEY, *Assistant to the Editor*.

Published bimonthly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Second class postage paid at Chicago, Ill., and at additional mailing offices. Reproduction of any part of this publication is prohibited unless special permission is given by the publishers and the authors. Subscription price: $15.00 per year in the United States; Canada, $15.50; Pan American countries, $16.50; foreign countries, $17.00. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $5.00 per year added to the annual dues of $25.00. Single copies, $2.50. Address all correspondence to American Veterinary Medical Association.

Change of address: Send new address (and moving date) 45 days before moving. Include old address as it appeared on previous issue (if possible send label). Do not depend on the post office to forward the JOURNAL.

DIFCO

Antisera *and* Antigens

E. coli
ENTEROPATHOGENIC
Salmonella Shigella Klebsiella
Bethesda-Ballerup

Proteus
RICKETTSIAL
Brucella Tularense

Streptococci Pneumococci Meningococci

B. pertussis C. albicans Trichinella

H. influenzae Leptospira

C. Protein Antiserum and Standard

Infectious Mononucleosis Antigens and Standards

Fluorescent Tagged Antibodies and Reagents

POTENT STABLE SPECIFIC

Descriptive literature on request

Specify

DIFCO LABORATORY PRODUCTS
BIOLOGICS CULTURE MEDIA REAGENTS

FALL FRESHENERS

RESULTS OF VACCINATION OF CATTLE WITH STAPHYLOCOCCAL BACTERIN-TOXOID

	No. of cows	Av. normal antitoxin titer	Av. antitoxin titer 1 mo. after 2nd injection	No. of infected quarters at time of vaccination	No. of infected quarters after 8 mo.	Acute infections during 8 mo.
Vaccinated*	45	12 I.U./ml.	33 I.U./ml.	46	40	10
Control	35	10 I.U./ml.	10 I.U./ml.	29	33	19

*Vaccination procedure: two injections of 10 ml. of bacterin-toxoid at four-week intervals.

STAPHYLOCOCCUS AUREUS TOXOID, SLANETZ STRAIN No. 7 is available in vials of 50 cc. and 250 cc.

Fall fresheners are doubly in need of the best possible protection against staphylococcal mastitis.

(1) Immediately following calving, the cow reaches her highest peak of production, and (2) during the fall and winter stabling period the highest incidence of mastitis occurs.

The seriousness of the problem of staphylococcal mastitis is steadily increasing as pointed up by the reports of many veterinarians who state that from 40 to 60 per cent of the cases they are called on to treat can be attributed to the persistent staphylococcic organism.

Vaccination with Cyanamid Staphylococcus Aureus Toxoid Slanetz Strain No. 7 should preferably be carried out prior to freshening, although this program also can be carried out effectively and safely at any point during the lactation period, as well as while the cow is dry. Procedure calls for 5 cc. intramuscularly, repeated in one month and annually thereafter.

Complete vaccination of the entire milking herd has proved effective in preventing the spread of staphylococcal mastitis to noninfected cows and preventing acute cases in cows already infected.

Cyanamid Staphylococcus Aureus Toxoid was developed and tested by Dr. L. W. Slanetz and his associates at the University of New Hampshire and has established a record of high efficacy in professional use by veterinarians.

Cyanamid has developed a complete, highly practical and effective mastitis control program around Staphylococcus Aureus Toxoid. This program attacks the mastitis problem in all its phases—and requires the dairyman to utilize the continuing supervision, guidance and professional services of his veterinarian.

If you would like the procedure booklet on the Cyanamid Mastitis Control Program, please write to Veterinary Professional Service Department, American Cyanamid Company, Princeton, New Jersey.

CYANAMID

STAPHYLOCOCCUS AUREUS TOXOID

(Continued from page 953)

Studies on Meningiomas in Cats 1030
H. Luginbuhl

Contrast Encephalography and Ventriculography in the Dog—Preliminary Studies .. 1041
B. F. Hoerlein and M. F. Petty

The Chemical Analysis of Normal Canine Prostatic Fluid 1057
H. Rosenkrantz, J. Langille, and M. M. Mason

Further Studies on Prophylactic Therapy Against the Developing Stages of *Dirofilaria immitis* Before Reaching the Canine Heart 1066
S. Kume, I. Ohishi, and S. Kobayashi

Use of Cell Culture-Adapted Canine Distemper Virus in the Complement-Fixation Test 1069
D. T. Karzon, J. H. Gillespie, and R. H. Bussell

A Technique for Concentrating *Mycobacterium paratuberculosis* Present in Intestinal Mucosa 1074
A. B. Larsen and R. S. Merkal

Association Among Members of the Genus *Metastrongylus* Molin, 1861 (Nematoda: Metastrongylidae) 1077
S. A. Ewing and A. C. Todd

Studies on a Skin Test for Leptospirosis 1081
D. C. Blenden, H. S. Goldberg, and P. Kuppuswamy

Direct Cultural Methods for the Isolation of Leptospires from Experimentally Infected Guinea Pigs 1085
R. W. Menges and M. M. Galton

Estimation of Cardiac Output by the Direct Fick Technique in Domestic Animals with Observation on a Case of Traumatic Pericarditis 1093
C. M. Stowe and A. L. Good

Uterine Healing After Cesarotomy in Goats 1097
R. P. S. Tyagi and W. V. Lumb

Critical Tests with the Organic Phosphate Insecticide, Dimethoate, Against *Gastrophilus spp.* in the Horse, with Observations on Its Anthelmintic Action 1106
J. H. Drudge, S. E. Leland, Jr., Z. N. Wyant, G. W. Elam, and E. T. Lyons

Preliminary Findings Concerning the Formalin Inactivation of Herpes B Virus 1112
J. E. Prier and N. R. Goulet

The Leukotaxic Properties of Uterine Exudates as Related to the Endocrine-Controlled Uterine Defense Mechanism 1177
H. W. Hawk, G. D. Turner, and J. F. Sykes

(Continued on page 959)

(Continued from page 958)

Scientific Notes

Electron Microscopic Observations of a Mucoid Stain of *Vibrio fetus*—*H. J. Werner, H. Levy, and B. O. Spurlock*..... 1121

The Intestinal Fluke *(Fasciolopsis buski)* in a Monkey—*H. A. Hartman*..... 1123

The Protozoan Parasite, *Klossiella equi*, in the Mexican Burro—*H. A. Hartman* 1126

Research Tools..... 1129

Index..... 1133

Classified Advertisements

The American Journal of Veterinary Research accepts classified advertisements from graduate veterinarians seeking veterinary technological positions with colleges, universities, and other institutions such as industrial and commercial research laboratories.

Also, classified advertisements are accepted from institutions and other organizations which may be seeking veterinary bacteriologists, parasitologists, pathologists, or other veterinary technological personnel.

This service is intended to facilitate the placement or finding of personnel with specialized training in veterinary medical science.

The rate for these classified advertisements is $5.00 for the first 25 words and 25 cents for each additional word; a $1.00 charge is made for use of a box number.

The closing date for classified advertisements in this Journal (a bi-monthly) is the 20th of the month two months preceding date of issue.

Wanted: D.V.M.-Ph.D. in bacteriology for diagnostic laboratory. Opportunity for research in diagnostic methods. New laboratory, excellent equipment. Send curriculum vitae to Dr. Wm. L. Sippel, Box 847, Kissimmee, Fla.

at calving time...

for retained placenta FUREA®

Bolus Veterinary

Prevents or controls uterine infection associated with retained placenta • results in higher conception rates • fewer services per conception. Bottle of 25 boluses.

for calf scours ENTEFUR®

Bolus Veterinary, Small

Often effective within 12 hours • diarrhea halted in an average of less than 3 days • recovery, even in some cases of seemingly moribund calves with bloody feces, surprisingly rapid and complete. Bolus of 3.3 Gm., boxes of 24 and 500.

Available through your professional veterinary distributor. / References and reprints on request.

EATON LABORATORIES, Division of The Norwich Pharmacal Company, NORWICH, N.Y.

American Journal of Veterinary Research
Established 1940
COPYRIGHT 1961 BY AMERICAN VETERINARY MEDICAL ASSOCIATION
VOL. XXII NOVEMBER, 1961 No. 91

Oxalate Formation in Moldy Feedstuffs as a Possible Factor in Livestock Toxic Disease

Benjamin J. Wilson, M.S., Ph.D., and Christina H. Wilson, R.N.

SUMMARY

Sixteen strains of fungi, including representatives of toxigenic species, were studied for toxigenicity in relation to oxalate formation. In addition to those previously identified as oxalate-positive, three additional species, *Aspergillus luchuensis*, *Aspergillus flavus*, and an unidentified *Aspergillus sp.* were found to be oxalate-positive and lethal for mice. Mice injected intraperitoneally with culture filtrate or culture-extracted oxalate salts developed central nervous system signs and lethal effects characteristic of acute oxalate poisoning.

Inoculation of several feedstuffs with individual oxalate-forming species gave rise to significant quantities of this organic acid. Analyses of infected wheat, oats, and Bermuda grass hay revealed oxalate well in excess of that theoretically required to precipitate all calcium present, under optimal conditions. Growth on various preparations of corn feeds resulted in very small increments of oxalate in most instances. *Aspergillus niger* growth produced marked increases of oxalate in hay and oats at both 26 C. and 35 C., beginning three days after inoculation and continuing for at least 12 to 15 days. Moldy grain corn, causing hepatitis x disease in dogs, contained normal quantities. In most feeds, a direct relationship was evident between the quantity of oxalate produced by mold contamination and the quantity of calcium present, suggesting a chemical combination of these substances within the feed.

Some individual and mixed feed preparations supported fungus growth and oxalate formation at levels not potentially capable of effecting a complete calcium deficiency. However, quantities produced were sufficient theoretically to precipitate a large portion of the total calcium, given optimal conditions for chemical combination.

Mice developed signs of severe malnutrition and died within a few days after being placed on a diet of moldy feed containing excess oxalate. Lightly infected feed caused weight losses but no deaths over a 30-day period. Survivors that were returned to regular diet recovered in most cases. Rabbits and guinea pigs fed moldy food not containing excess oxalate lost much weight but at no time had overt signs of acute oxalate poisoning.

Received for publication Nov. 25, 1960.
From David Lipscomb College, Nashville, Tenn., where Dr. Wilson is associate professor of biology and Mrs. Wilson is biological research technician.
Supported by grants from the Medical Science Research Foundation, Stanford University, Calif., and Texaco, Incorporated, Beacon, N.Y.
The authors thank Dr. Frank R. Blood, School of Medicine, Vanderbilt University, for valuable information and references on oxalic acid poisoning.

INTRODUCTION

During a preliminary study of certain toxigenic fungi,[14] crystalline substances with lethal properties for mice and rabbits

were extracted from culture filtrates of *Aspergillus luchuensis*. Intradermal injection in guinea pigs resulted in formation of a lesion with a dark necrotic center, while topical application elicited no effect. Chemical analysis of these materials indicated oxalic acid principle as the water soluble salt. The intraperitoneal lethal dose for 50% ($IPLD_{50}$) of 20- to 25-Gm. mice was about 3 mg. Death, preceded by tetany and convulsions, usually occurred within five to 30 minutes after injection.

Oxalate production by various species of commonly encountered fungi was known as early as 1887.[11] Extensive experiments by Wehmer[29] on factors affecting oxalate formation in culture mediums suggested the desirability of patenting the biologic process for possible commercial application. Chemical methods for obtaining oxalic acid and oxalate salts soon proved more practical.

The possibility that this substance is elaborated in dangerous quantities in moldy feeds has not been seriously considered. Currie and Thom[8] speculated that quantities significant in food chemistry might be produced only by one species with which they worked, *Penicillium oxalicum*. In this respect *P. oxalicum* compared favorably with *Aspergillus niger*, then considered the greatest oxalate producer.

Early studies had demonstrated that various filamentous and fleshy fungi were capable of degrading food substrates to oxalate. The filamentous organisms included *A. niger, Botrytis cinerea, Rhizopus nigricans, Phycomyces nitens, Pilobilus crystallinus*, and *Mucor mucedo*.[11] Twenty-three species of *Penicillium* were reported oxalate-positive by Currie and Thom.[8]

A possible relationship between mold oxalate and various epizootics of mycotoxicosis is supported by identifying certain clinical signs seen in these epizootics,[24,30] with those of halogeton poisoning and other hypocalcemic states in animals.[15,22,25] Such a relationship, if established, would indicate that oxalate is yet another toxic substance to be included with those described in certain cases of moldy feed poisoning.[5,6,9,10]

The following laboratory experiments were performed to obtain preliminary information on this possibility.

Experimental Procedure and Results

Oxalate-Producing Properties and Toxigenicity of Available Stock Strains of Fungi.—Several collected strains of fungi, including representatives of species reportedly toxigenic, were screened for toxigenicity and ability to form oxalate. Each was inoculated into flasks of Sabouraud-dextrose broth, pH 6.0, containing 0.05M sodium acetate. Experience had shown this medium supported excellent growth and oxalate production by *A. niger* and *A. luchuensis* in both static and shaking cultures. All cultures were incubated with shaking at 26 C. for a period of 15 days. One-milliliter samples of the different culture filtrates were analyzed by precipitation of the oxalate with calcium acetate at pH 4.0 and titration with 0.01N potassium permanganate at 70 C. Toxicity was determined by injecting, intraperitoneally, 1 ml. of each filtrate into 6 white mice weighing 20 to 25 Gm.

The results of this preliminary survey and other data relative to each species are shown (Table 1). Filtrates of the oxalate-producing aspergilli caused death in mice within a period of a few minutes to four days. In every case of fungus oxalate poisoning, typical signs were noticed 5 to 30 minutes after injection. These included tremors of the head, wobbling gait, and general incoordination of movements. Those dying within the first hour had convulsions culminating in hyperextension of the limbs, arched back, apnea, and respiratory paralysis. These terminal attacks were sometimes evoked by moderate stimuli and were similar to those of strychnine poisoning. Culture filtrates containing the most oxalate killed within a few minutes after injection, while those with smaller amounts required correspondingly longer periods of time.

Previous work with the *A. niger* and *A. luchuensis* strains has shown that toxicity resides principally, if not solely, in oxalate salts produced in large quantities under optimal culture conditions. Certain strains of *Penicillium rubrum, Penicillium citrinum*, and *Aspergillus clavatus* have been known to produce toxic antibiotic substances which may account for the effects observed in this study. Culture filtrates of the other potentially toxigenic species, however, did not manifest any untoward effect in mice under these experimental conditions.

Oxalate Formation as a Result of Mold Growth in Various Livestock Feedstuffs.—Fifty-gram quantities of whole grain cereals were placed in separate 2-liter Erlenmeyer or Fernbach flasks, 100 to 150 ml. of water was added, and the flasks were autoclaved for 45 minutes. Separate samples of different feeds were inoculated respectively with the various fungus species under

consideration. The flasks were shaken once daily during the first week to distribute the developing mycelium and spores throughout the grains. Samples of Bermuda grass hay were dispensed in 20-Gm. quantities. Other test feed materials were prepared in quantities adequate for calcium and oxalate assays and animal feeding tests. Incubation was carried out at 26 C. (79 F.) for 15 days. Following this period, contents of the flasks were dried for 48 hours at 90 C., and ground to 2- to 3-mm. particles. A 2.5-Gm. sample was extracted from each fungus-feed preparation, and the total oxalate was determined according to the method of Moir.[20] To determine calcium content, 2-Gm. quantities were ashed and extracted with 0.25N HCl. Duplicate portions of this extract were assayed by the method of Clark and Collip.[7]

Table 2 gives the total calcium and oxalate found in normal and contaminated wheat, oats, Bermuda grass hay, and corn. In wheat, oats, and hay, growth of the aspergilli significantly increased the oxalate content. *Myrothecium verrucaria* cultures on wheat and hay, however, resulted in reduction of the original oxalate content, as indicated by no substantial change and a reduction, respectively, in the assay figures. Utilization of weight-contributing substrate without oxalate degradation otherwise would have resulted in a relative increase in oxalate.

Uninoculated Bermuda grass hay had the highest concentrations of calcium and oxalate of any normal feed tested. Coincidentally, growth of the various oxalate-producing aspergilli on this substance resulted in the greatest increments recorded.

TABLE 1—Oxalate Production and Toxicity Data on Collected Species of Fungi Grown in Sabouraud Dextrose-Acetate Broth, 15 Days at 26 C.

Organism	Reports of species toxicity (references)	Mg. oxalate as $H_2C_2O_4$ per ml. filtrate	Mouse i.p. injection results
*Aspergillus chevalieri** QM 1974	Toxic [6]	Negative	Negative
*Aspergillus clavatus** QM 6884	Toxic [16, 28]	Negative	Illness: recovered
*Aspergillus flavus** QM 6738	Toxic [5]	0.96	Death
*Aspergillus luchuensis** QM 5565	Toxic [14]	2.05	Death
Aspergillus niger	Unknown	7.44	Death
*Aspergillus oryzae** QM 6736	Non-toxic [6]	Negative	Negative
*Aspergillus sp.*** (Alabama moldy corn)	Unknown	Negative	Negative
*Aspergillus sp.**** (Florida moldy corn)	Unknown	1.92	Death
*Myrothecium verrucaria** QM 406	Toxic [14]	Trace	Negative
*Penicillium rubrum** QM 124 W-2	Toxic [5]	Negative	Death
*Penicillium citrinum** QM 6748	Toxic [1, 14]	Negative	Death
*Penicillium sp.*** (Alabama moldy corn)	Unknown	Negative	Negative
Mucor genevensis (plus strain)	Unknown	Negative	Negative
Rhizopus nigricans	Unknown	Negative	Negative
Sporodesmium bakeri† Strain "G"	Toxic [27]	Negative	Negative
Stachybotrys atra‡ Forgacs 1006	Toxic [9]	Negative	Negative

* Cultures supplied through the courtesy of Dr. Emory Simmons, U.S. Army Quartermaster, Research and Engineering Command, Natick, Mass.

** Isolated from Alabama moldy corn sample producing "hepatitis x" in dogs. Sample provided by Dr. W. S. Bailey, School of Veterinary Medicine, Auburn University, Auburn, Ala.

*** Predominant organism in hepatotoxic moldy corn from Florida field near site of moldy corn disease epizootic in swine. Procured by Dr. W. S. Bailey of Auburn University.

† Isolated from hepatotoxic grass and supplied through the courtesy of Dr. R. H. Thornton, Taita Experimental Station, Lower Hutt, New Zealand.

‡ Ether extract of hay culture dermonecrotic for guinea pigs and rabbits. Culture provided by Dr. R. S. Lincoln, Fort Detrick, Md. (ex Forgacs).[9]

Infected whole grain corn provided only small amounts of oxalate, if there were any increase at all. The largest quantity in this food was found in the sweet corn chop material that included husk along with cob and kernels.

Table 2 also contains data on a ground corn sample responsible for a moldy corn as the principal mineral regulating soluble oxalate levels in infected feeds. Wehmer [29] found that cations such as NH_4^+, Na^+, K^+, and Ca^{++} in culture mediums trapped oxalate ions and permitted their accumulation.

With the few exceptions mentioned for corn products, oxalate produced was in excess of that empirically required to pre-

TABLE 2—Calcium and Oxalate Content of Feedstuffs Inoculated with Spores of Various Fungi and Incubated 15 Days at 26 C.

Fungus	Calcium*	Oxalic acid*	Oxalic acid/calcium ratio	Excess** oxalic acid
WHEAT (WHOLE GRAIN)				
Normal	0.04	0.13	3.3	0.04
Infected				
M. verrucaria	0.06	0.13	2.2	none
A. luchuensis	0.05	0.63	12.6	0.52
A. niger	0.07	0.73	10.5	0.58
OATS (WHOLE GRAIN)				
Normal	0.07	0.17	2.4	0.02
Infected				
A. luchuensis	0.10	1.43	14.3	1.20
A. niger	0.09	1.13	12.6	0.93
BERMUDA GRASS HAY				
Normal	0.73	1.34	1.8	none
Infected				
M. verrucaria	0.80	0.31	0.4	none
A. luchuensis	0.80	3.26	4.0	1.44
A. niger	0.82	5.40	6.6	3.56
A. flavus	0.96	4.25	4.4	2.09
CORN				
Canned, yellow sweet				
Normal	0.02	trace	.....	none
Infected				
A. luchuensis	0.03	0.13	4.31	0.07
Corn chop (sweet corn, chopped ear with husk)				
Normal	0.04	trace	.....	none
Infected				
A. niger	0.07	0.44	6.3	0.28
Hard corn (whole grain)				
Normal	0.01	0.12	25.0	0.11
Infected				
A. luchuensis	0.01	0.08	13.3	0.07
A. niger	0.01	0.28	46.7	0.27
Toxic, moldy corn (yellow); associated with "hepatitis "X" of dogs	0.02	0.13	6.5	0.08

* Expressed as per cent of feed dry weight: Calcium as Ca, oxalate as anhydrous acid.
** Per cent oxalic acid in excess of quantity theoretically required to precipitate total calcium.

disease epizootic of swine in Alabama. Hepatitis x was produced in dogs force-fed portions of this lot.[2] In view of the small oxalate content, a negative relationship is indicated for this principle and the hepatic disease.

In corn, as with the other feedstuffs tested, preparations with highest calcium content gave rise to most oxalate. This observation suggests that available plant calcium may combine with oxalate as it is produced by the fungus, thereby serving cipitate, under optimal conditions, all available calcium of the infected feeds listed (Table 2). These "excesses" (presumably water-soluble salts) were greatest in the Bermuda grass hay samples.

Table 3 lists additional feed preparations in which infection with *A. niger* resulted in significant oxalate accumulations. However, with the exception of rye seed, oxalate was not produced in sufficient quantities to bind all available calcium as insoluble calcium oxalate.

TABLE 3—Calcium and Oxalate Data on Various Normal and Aspergillus niger Infected Feed Preparations

Feed	Calcium*	Oxalic acid* equivalent	Excess calcium*	Potential calcium decrease**
OATS-CORN-MOLASSES HORSE FEED (CALCIUM FORTIFIED)				
Normal	0.23	neg.	0.23	70
Infected	0.28	0.49	0.07	
RYE GRASS (PERENNIAL VARIETY)				
Normal	0.53	neg.	0.53	none
Infected	0.60	0.09	0.57	
RYE (SEED)				
Normal	0.03	trace	0.03	100
Infected	0.04	0.42	none	
JOHNSON GRASS				
Normal	0.69	trace	0.69	87
Infected	0.89	1.82	0.09	
RABBIT DIET (PELLETIZED, CALCIUM FORTIFIED)				
Normal	1.22	neg.	1.22	71
Infected	1.85	3.38	0.36	
MOUSE DIET				
Normal	0.38	0.19	0.30	none
Infected	0.40	0.21	0.31	
GUINEA PIG DIET (PELLETIZED, CALCIUM FORTIFIED)				
Normal	ND	ND	.....	99
Infected	3.0	6.7	0.02	

* Expressed as per cent of feed dry weight.
** Per cent reduction of available calcium assuming chemical combination with fungus oxalate.

Time and Temperature Effects on Oxalate Formation by Aspergillus niger Growing on Oats and Hay.—Several 4-Gm. samples of ground oats and chopped Bermuda grass hay were placed in 125-ml. and 250-ml. Erlenmeyer flasks, respectively. Ten milliliters of water was added to each flask before autoclaving. All except control flasks were inoculated with 1 ml. of a standard aqueous suspension of *A. niger* spores. Half of each group was held at 26 C., while the remaining flasks were incubated at 35 C. Small additions of water were made during incubation to replace the moisture lost by evaporation. One flask from each incubation temperature group was removed at intervals of 3, 6, 9, 12, and 15 days. Each sample was processed and assayed for total oxalate as previously outlined. Calcium determinations were made on ashed samples as before.

Table 4 lists calcium and oxalate content of the samples used in this experiment. A marked increment occurred within three days and continued for 12 to 15 days. No significant benefit of the higher temperature on oxalate production was evident in the hay but, with oats, 35 C. appeared to favor higher values at every time interval. Calcium increased relatively as metabolizable substrates were converted to oxalate and other degradation products. Prolonged incubation might have increased the oxalate even further, since in two of the feed-temperature series values rose to new heights on the final day of incubation. Early fungus oxalate studies established that formation continued as long as suitable substrate and cations were present in culture mediums.[29]

Preliminary Feeding Experiments on Laboratory Animals Employing Moldy Feed Preparations.—Limitations of facilities made it impossible

TABLE 4—Effect of Different Growth Periods and Two Temperatures on Calcium and Oxalate Contents of Ground Oats and Bermuda Grass Hay Inoculated with Aspergillus niger Spores

	Oats				Hay			
	26 C.		35 C.		26 C.		35 C.	
Growth (days)	Calcium*	Oxalate* as $H_2C_2O_4$	Calcium	Oxalate as $H_2C_2O_4$	Calcium	Oxalate as $H_2C_2O_4$	Calcium	Oxalate as $H_2C_2O_4$
Control	0.07	0.15	0.07	0.18	0.79	1.34	0.73	1.29
3	0.07	0.86	0.07	0.93	0.80	3.62	0.81	3.93
6	0.08	0.91	0.09	1.40	0.83	4.64	0.82	4.46
9	0.08	1.13	0.09	1.43	0.82	4.06	0.82	4.08
12	0.08	1.26	0.09	1.41	0.83	4.62	0.83	4.63
15	0.09	1.02	0.10	1.53	1.06	5.58	0.83	4.00

* Expressed as per cent of feed dry weight.

to carry out the type of nutrition studies required to assess several important aspects of moldy feed poisoning in laboratory animals. Growth of oxalate-positive fungi on feeds of necessity causes a variety of chemical changes in the various organic substances. Such changes might include vitamin depletion and loss of caloric value as well as calcium precipitation.

The first feeding tests were made with a group of 6 white mice, each weighing approximately 30 Gm. These were given *A. niger* infected wheat ad libitum throughout the period of the experiment. This preparation contained 0.07% calcium and 0.73% oxalic acid (See Table 2).

The animals first refused the food but began to eat sparingly on the second day. By the third day, 2 of the 6 mice were found dead and partially eaten by cage mates. Both carcasses had hyperextended hind legs and severely arched backs. The remaining mice lost much weight but did not show tremors or convulsions when stimulated by rough handling. The following day, 1 more mouse was found dead. The remaining 2 made uncomplicated recoveries when given normal diet. The quantity of infected food consumed was about double that of uninfected wheat given 6 control mice. Controls developed no abnormalities throughout the period of testing.

A repetition of this experiment, using oats infected with *A. niger* (see Table 2) as food for 12 mice, resulted in a death pattern similar to that above. Six surviving mice were then given lightly infected oats for one month. No further deaths occurred, although the mice remained at low weight. Again, the quantity of feed consumed was much larger than that of controls on normal oats. When heavily contaminated feed was reintroduced, the remaining mice became more emaciated, and the normal red eye color diminished. Upon microscopic examination of urinary sediments from randomly selected moribund mice, an abundance of calcium oxalate crystals was found. One by one the mice became weak and succumbed over a period of several days.

Commercially prepared mouse diet, containing skim milk and calcium compounds, was thoroughly wetted and inoculated with *A. niger* spores. After 14 days of incubation at room temperature, the oxalate content had risen only from 0.19 to 0.21%, while the final calcium content was 0.4% (Table 3). Mice consumed this food, reluctantly at first, with no apparent detrimental effects over a period of six weeks. The available calcium content and the caloric value of this high-lipid (11% crude fat) feed apparently were not seriously impaired.

Guinea pig and rabbit pellet diets were similarly dampened and inoculated with *A. niger* spores. After 14 days of incubation, these foods were fed to animals of the respective groups. No gross abnormalities were noticed in adult animals, except for marked weight losses and diminished activity. Immature guinea pigs did not grow normally, as did control animals. A drop in food caloric value was suggested by an approximate doubling of the usual ad libitum consumption by each animal. Analysis (Table 3) revealed a large amount of oxalate but no theoretical excess.

Discussion

Both oxalate production by various species of fungi and moldy feed poisoning have been recognized for several decades.[4,11] However, a search of foreign as well as American scientific literature revealed no references indicating a relationship between these two phenomena.

Many species of fungi prevalent in various environments are capable of degrading basic food substrates to oxalate. Three additional aspergilli, including two representing toxigenic species, also were found to possess this metabolic property. One of these was *A. flavus*, of which both toxic and nontoxic strains have been isolated from disease-producing feeds.[5,6] The second species, *A. luchuensis*, was found by Harkness *et al.* to cause rapid death in rabbits when the filtrate was injected intravenously. The third was an unidentified species isolated from hepatotoxic moldy Florida corn.

Various reports of livestock disease, including some associated with toxic feedstuffs, have described signs similar to those of oxalate poisoning. These include gastritis, albuminuria, urinary calculi, unsteady gait, falling with inability to rise, dementia, depression, hyperexcitability, tetany, paralysis, and convulsions leading to death. In cases of winter tetany of cattle[18,19] where clinical tests were made, a hypocalcemia and hypomagnesemia were discovered; injections of therapeutic preparations of calcium and magnesium evoked a prompt cessation of signs. This information leads one to consider oxalate from

moldy feeds, especially hay, as the causative agent in some of these winter illnesses. No such relationship, however, is indicated in grass and wheat tetany, since the presence of moldy grass is infrequently noticed and the oxalate content of plant samples is not significantly elevated.

Mershon [18] reported that the incidence of bovine tetany in Garrett County, Maryland, reaches a peak late in winter. Commenting on this, he states,* "In some instances over-ripe timothy or mixed hay was fed following poor harvesting conditions that resulted in wetting the hay. Mold was frequently observed, and it was believed that such hay was low in nutrients and minerals as well as being unpalatable, and that the cows were effectively starved in regard to some nutrients."

The foregoing experiments demonstrated that the oxalate-positive species of *Aspergillus* employed were capable of producing relatively large quantities of this organic acid in some feedstuffs at 26 C. *Aspergillus niger* accumulated high levels in oats and hay at both 26 C. and 35 C. (79 F. and 95 F.). Appreciable oxalate production at both of these temperatures is important, since they cover a range that includes ambient summer temperatures in much of the temperate zone. *Aspergillus niger* and many other oxalate-producing species are probably capable of comparable metabolic activity throughout a large portion of their temperature growth ranges.

The small quantity of oxalate produced by infection of different corn feeds and the low level in hepatotoxic corn samples do not indicate a relationship of oxalates to moldy corn toxicoses. However, the small quantity of available calcium normally present in corn makes advisable supplementation of its feeding with other substances of higher calcium content.[21] Thus, small quantities of oxalate might have more adverse effect in animals such as swine, which normally require high calcium intake.[3]

The danger in feeding moldy Bermuda grass hay, however, is readily apparent. For example, a horse whose feed might consist principally of this ration would consume daily up to 300 to 350 Gm. of oxalic acid as soluble salts in each 20 lb. of hay.

Adverse effects of high oxalate foods have been demonstrated by several investigators. Garcia-Rivera and Morris [12] suggested that low milk output by Puerto Rican cattle may be traced to regular grazing on grasses containing more than 1.6% oxalic acid. One species of grass, *Pennisetum merkerii*, contained about 5% total oxalate and was responsible for a negative calcium balance in cattle.* Robertson and co-workers [23] showed that 2% oxalic acid in chick rations was lethal, while growth was depressed by a diet containing 1.0% oxalic acid and 0.9% calcium. The depression was overcome when the calcium level was increased to 1.6%. Using laboratory rats in experimental feeding tests, Kohman [16] concluded that spinach, although relatively high in calcium, supplies none of this element to the body and renders unavailable a considerable amount of that in other foods.

As to the mechanism of oxalate toxicity in ruminants, Talapatra *et al.*[26] found that ingested soluble oxalates were degraded to alkali carbonates before reaching the true stomach; these compounds were believed to be responsible for a severe alkalosis interfering with calcium absorption. Preformed calcium oxalate was excreted unchanged in the feces.

Whatever the mechanism of action, all available evidence indicates that continued ingestion of excess oxalate inevitably leads to a negative calcium balance in many animals. Absorption of sublethal quantities of soluble oxalates from the gastrointestinal tract can create calcium oxalate in body tissues, leading to calculi formation in kidney tubules and resultant renal damage.[13]

Phytic acid of whole wheat and oats also is capable of binding dietary calcium and rendering it unavailable to the organism.[17] Therefore, one might presuppose an additive effect of this substance with oxalate.

In addition to a possible lowering of feed calcium availability, fungus growth necessarily decreases the caloric value of feed to a marked extent. The increased consumption of moldy food noticed in preliminary feeding trials supports this idea. To compensate for a caloric deficiency, the amount of feed consumed is increased, thereby augmenting the intake of oxalate. Alteration of the calcium:phosphorus intake ratio and destruction of the food vita-

* Mershon, M. M.; Maryland Live Stock Sanitary Service Laboratory, Bel Air, Md.: Personal communication, Oct. 26, 1960.

* Garcia-Rivera, J.; University of Puerto Rico, College of Agriculture and Mechanic Arts, Mayaques, Puerto Rico: Personal communication, Sept. 28, 1960.

min content are also to be considered in the possible mechanisms of oxalate poisoning.

The animal feeding tests reported do not provide information on the mechanisms of malnutrition or poisoning described. Extended and carefully controlled feeding experiments employing various livestock need to be carried out where all factors of cause and effect can be properly evaluated by personnel trained in the fields of animal nutrition and pathology.

References

[1] Ambrose, A. M., and De Eds, F.: Some Toxicological and Pharmacological Properties of Citrinin. J. Pharm. & Expti. Therap., 88, (1946): 173–186.

[2] Bailey, W. S., and Groth, A. H.: The Relationship of Hepatitis X of Dogs and Moldy Corn Poisoning of Swine. J.A.V.M.A., 134, (June 1, 1959): 514–516.

[3] Blaxter, K. L.: The Utilization of Minerals, Vitamins, and Other Constituents of Grass. Brit. J. Nutr., 6, (1952): 110–116.

[4] Buckley, S. S., and MacCallum, W. G.: Acute Hemorrhagic Encephalitis Prevalent Among Horses in Maryland. Am. Vet. Rev., 25, (1901): 99–102.

[5] Burnside, J. E., Sippel, W. L., Forgacs, J., Carll, W. T., Atwood, M. B., and Doll, E. R.: A Disease of Swine and Cattle Caused by Eating Moldy Corn. II. Experimental Production with Pure Cultures of Molds. Am. J. Vet. Res., 18, (Oct., 1957): 817–824.

[6] Carll, W. T., Forgacs, J., and Herring, A. S.: Toxicity of Fungi Isolated from a Food Concentrate. Am. J. Hyg., 60, (1954): 8–14.

[7] Clark, E. P., and Collip, J. B.: A Study of the Tisdall Method for the Determination of Blood Serum Calcium with a Suggested Modification. J. Biol. Chem., 63, (1925): 461–464.

[8] Currie, J. N., and Thom, C.: An Oxalic Acid-Producing *Penicillium*. J. Biol. Chem., 22, (1915): 287–293.

[9] Forgacs, J., Carll, W. T., Herring, A. S., and Hinshaw, W. R.: Toxicity of *Stachybotrys atra* for Animals. Ann. New York Acad. Sci., Ser. II, 20, (1958): 787–808.

[10] Forgacs, J., Carll, W. T., Herring, A. S., and Mahlandt, B. G.: A Toxic *Aspergillus clavatus* Isolated from Feed Pellets. Am. J. Hyg., 60, (1954): 15–26.

[11] Foster, J. W.: Chemical Activities of Fungi. Academic Press, New York, (1946): 326–350.

[12] Garcia-Rivera, J., and Morris, M. P.: Oxalate Content of Tropical Forage Grasses. Science, 122, (1955): 1080–1090.

[13] Goodman, L. S., and Gilman, A. Z.: Pharmacological Basis of Therapeutics. 2nd ed. Macmillan Co., New York, (1955): 832–833.

[14] Harkness, W. D., Loving, W. L., and Hodges, F. A.: Pyrexia in Rabbits Following the Injection of Filtrates of Typical Mold Cultures. J. Am. Pharm. A., Sci. ed., 39–40, (1950–51): 502–504.

[15] Hughes, P., and Cornelius, C. E.: An Outbreak of Grass Tetany in Lactating Beef Cattle. Cornell Vet., 50, (1960): 26–33.

[16] Kohman, E. F.: Oxalic Acid in Foods and Its Behavior in the Diet. J. Nutr., 18, (1939): 233–246.

[17] McCance, R. A., and Widdowson, E. M.: Phytic Acid. Brit. J. Nutr., 1–2, (1947–1949): 401–403.

[18] Mershon, M. M.: Tetany in Cattle on Winter Rations. I. A Clinical Report. J.A.V.M.A., 132, (May 1, 1958): 396–400.

[19] Mershon, M. M.: Tetany in Cattle on Winter Rations. II. Stresses and Mineral Metabolism. J.A.V.M.A., 135, (1959): 435–439.

[20] Moir, K. W.: The Determination of Oxalic Acid in Plants. Queensland J. Agric. Sci., 10, (1953): 1–3.

[21] Morrison, F. B.: Feeds and Feeding. 22nd ed. Morrison Publishing Co., Clinton, Iowa (1959): 420.

[22] Redmond, H. E.: Wheat Poisoning in Cattle. Southwest. Vet., March, 1950.

[23] Robertson, E. I., Brin, M., and Norris, L. C.: Use of Dehydrated Beet Leaves in Chick Rations. Poult. Sci., 26, (1947): 582–587.

[24] Rodriguez, J. A.: Differential Diagnosis of a Toxic Encephalitis Caused by *Aspergillus maydis*-Infected Fodder and Equine Encephalomyelitis. An. Soc. Rur. Agent, 69, (1945): 305–307.

[25] Sims, J. H., and Crookshank, H. R.: Wheat Pasture Poisoning in Cows. Texas Agric. Exper. Sta. Bull. 842, (1956): 6.

[26] Talapatra, S. K., Ray, S. C., and Sen, K. C.: Calcium Assimilation in Ruminants on Oxalate-Rich Diet. J. Agric. Sci., 37, (1948): 163–173.

[27] Thornton, R. H.: The Identification and Culture of *Sporodesmium bakeri*. Proc. New Zealand Soc. Anim. Prod., 19, (1959): 83–87.

[28] Waxman, S. A., Horning, E., and Spencer, E. L.: Two Antagonistic Fungi, *Aspergillus fumigatus* and *Aspergillus clavatus*, and Their Antibiotic Substances. J. Bact., 45, (1942): 233–248.

[29] Wehmer, C.: Chemical Activities of Fungi. Academic Press, New York, (1946): 327–333. Cited by Foster.[11]

[30] Zeller, R.: Poisoning with Toxic Moulds. Berl. Münch. tierärztl. Wchnschr., 68, (1955): 99–101.

SUMMARIO IN INTERLINGUA

Le Formation de Oxalato in Forrage Mucide Como Factor Possibile in Morbo Toxic de Bestial

Dece-sex racias de fungos, incluse representantes de species toxigene, esseva studiate pro lor toxigenicitate in relation al formation de oxalato. Esseva constatate que—a parte le species previemente identificate como oxalato-positive—tres noves esseva tal. Illos es *Aspergillus luchuensis*, *A. flavus*, e un non-identificate *A. sp.* Iste tres esseva letal pro muses. Muses subjicite a injectiones intraperitonee de filtratos ab le culturas o de sales oxalatic extrahite ab le culturas disveloppava signos de un affection del systema nervose central e effectos letal de character typic de acute invenenamento per oxalato.

Le inoculation de species individual a formation de oxalato ad in diverse typos de forrage resultava in le generation de significative quantitates de iste acid organic. Le analyse de inficite specimens de frumento, de avena, e de feno de herba de Bermuda revelava concentrationes de oxalato ben in ultra del concentrationes que es theoricamente requirite pro precipitar sub conditiones optimal omne le calcium presente. Le crescentia del fungos in varie preparatos de mais resultava, in le majoritate del casos, in solmente micrissime augmentos del oxalato. *A. niger* crescente in feno e avena produceva marcate augmentos de oxalato,

tanto a 26 como etiam a 35 C, a partir de tres dies post le inoculation e continuante durante al minus 12 a 15 dies. Mucide grano de mais, que causa "hepatitis X" in porcos e canes, esseva normal in su contento de oxalato. In le majoritate del specimens de forrage testate, un directe relation esseva evidente inter le quantitate de oxalato producite per le contamination mucoral e le quantitate de calcium presente. Iste constatation suggereva le occurrentia de un combination chimic de iste substantias intra le forrage.

Varie preparatos de forrage individual e mixte supportava un crescentia fungal e un formation consequente de oxalato a nivellos non potentialmente capace a effectuar un complete deficientia de calcium. Tamen, le quantitates producite esseva theoricamente sufficiente a precipitar un grande portion del calcium total, al minus in le presentia de conditiones optimal pro le combination chimic.

Muses disveloppava signos de sever malnutrition e moriva intra pauc dies post que illos habeva essite transferite a un dieta de forrage mucide a contento excessive de oxalato. Un levemente inficite forrage causava perdita de peso sed nulle morte intra un periodo de observation de 30 dies. Le superviventes que esseva retransferite a un dieta regular se restabliva in le majoritate del casos. Conilios e porcos de India nutrite con un dieta mucide sin contento excessive de oxalato perdeva multe peso sed non manifestava a ulle tempore signos patente de acute intoxication per oxalato.

Studies on Bovine Staphylococcic Mastitis. II. Characterization of Isolates

L. W. Fleming, M.A., and I. M. Paton, M.R.C.V.S.

SUMMARY

One hundred and one strains of staphylococci from bovine mastitis were studied. All but five of the strains were coagulase-positive, and only six did not ferment mannitol. Sixty-nine per cent of the isolates were sensitive to 25 typing phages, either singly or in various combinations. The largest single typing group contained 19 cultures sensitive to phage 42D (group IV). Twenty-nine cultures were lysed by phages of two or more of the broad typing groups.

Eighty-eight of the 101 strains were found to be sensitive to less than 1 unit of penicillin by the tube dilution method. Only two strains required as much as 25 units for inhibition.

All of the 52 strains examined for the production of *alpha* and *beta* toxin produced some *alpha* toxin. Forty-four per cent produced no demonstrable *beta* toxin. Group III and multigroup strains produced the most *alpha* toxin, whereas the miscellaneous strains (phage type 44A) appeared to be the best *beta* toxin producers. Group IV (42D) and miscellaneous strains produced fair amounts of both toxins. Typeable cultures in general produced more *alpha* and *beta* toxin than did the nontypeable cultures.

INTRODUCTION

In a recent publication,[6] a general discussion of bovine staphylococcic mastitis emphasized the need for formulation of immunizing agents for this costly disease. It was suggested that bacteriophage typing be used as the basis for selecting strains of staphylococci for the production of "multivalent" bacterin-toxoids. It would seem justified at this time to take advantage of the phage-type consistencies demonstrated in staphylococcic isolates from bovine mastitis. Despite the equivocal nature of group specificity, an immunizing agent would have more universal usefulness if it were formulated from strains representing several of the broad phage-typing groups.

This report will present the results of our characterization of 101 bacterial isolates collected from animals with mastitis in the United States and England. We were particularly interested in any relationships between the toxigenicity of isolates and their phage groups.

Materials and Methods

One hundred and one strains of *Staphylococcus aureus* were studied. The cultures were obtained from several different sources but represented isolates from animals with either acute or chronic bovine mastitis. Dr. E. H. Coles of Kansas State University, Manhattan, supplied many of the cultures, as did Dr. S. J. Edwards of the Compton Field Station at Berkshire, England. Miscellaneous collections of cultures were obtained from personnel at Pennsylvania State University and our own Clinical Medicine Department. Throughout the investigation, the cultures were maintained by monthly transfers on veal-infusion agar slants.

The cultures were typed by Dr. C. A. Hunter of the Kansas State Board of Health, using the technique of Blair and Carr[2] with slight modifications. A total of 25 phages was used; 19 were basic phages of the International series. For reference purposes, the individual phages are listed here in their broad groups. The phages enclosed by parentheses are the six additional phages used in typing our collection:

Group I —29, 52, 52A, 79, (80).
Group II —3A, 3B, 3C, 55.
Group III—6, 7, 42E, 47, 53, 54, 70, 73, 75, 77, (42B), (47C), (VA4), (81).
Group IV—42D.
Miscellaneous—(44A).

Cultures which were insensitive to the routine test dilutions of the typing phages were subsequently tested at 1,000 times the initial concentrations.

Fifty-two of the 101 cultures were studied to determine their ability to produce *alpha* and *beta*

Received for publication Oct. 24, 1960.

From Jensen-Salsbery Laboratories, Inc., Kansas City, Mo., where Mr. Fleming is bacteriologist, Research Department, and Dr. Paton is research director.

The authors thank Mrs. Margaret Davis for her technical assistance in this study.

exotoxins in a liquid medium. Thirty-milliliter quantities of brain-heart infusion broth (Difco), contained in 50-ml. Erlenmeyer flasks, were inoculated with 0.1 ml. of eighteen-hour broth cultures of the various strains. The flasks were placed in a converted desiccator jar which was sealed after flushing with a 20% CO_2 and 80% O_2 gas mixture. Incubation was at 37 C. for 64 hours on a shaker operating at 85 oscillations per minute. Incubation of the cultures was interrupted only to reflush the desiccator with fresh gas mixture at 24-hour intervals. Toxic supernatant fluids were collected after centrifugation of the cultures for 30 minutes at 2,500 r.p.m. Hemolytic titers of the fluids were determined by first making duplicate sets of serial twofold dilutions in 0.9% NaCl solution, beginning with a 1:5 dilution. One-milliliter quantities of the various test dilutions were mixed with equal volumes of a 2% suspension of thrice-washed rabbit or sheep erythrocytes. Donor animals were checked periodically for antitoxin titers and used as erythrocyte sources only so long as their serum contained less than 0.2 unit of *alpha* and *beta* antitoxin per milliliter. After three hours of incubation at 37 C., *alpha* toxin titers were read as the highest dilution at which any significant amount of hemolysis occurred. *Beta* titers were calculated as the arithmetical difference between the highest dilution which developed any hemolysis after three hours at 37 C. and that which developed hemolysis after overnight refrigeration. This method discounts the small amount of hemolysis of sheep erythrocytes after incubation at 37 C., which is probably caused by *alpha* toxin.

Sensitivities of the cultures to penicillin were determined by the tube dilution method. Bulk quantities of tryptose phosphate broth (Difco), containing 50.0 to 0.15 units of penicillin G potassium per milliliter of broth, were dispensed in 4-ml. amounts. A series of the antibiotic dilutions was inoculated with 0.1 ml. of a 10^{-3} dilution of an eighteen-hour broth culture of each staphylococcic strain. Following overnight incubation at 37 C., results were read and recorded as the minimum concentration of penicillin per milliliter of medium which would completely inhibit visible growth of the test culture.

Coagulase production and mannitol fermentation were determined by standard methods. Citrated rabbit plasma was diluted 1:4 in 0.9% NaCl solution and dispensed in 0.5-ml. amounts. An equal volume of an eighteen-hour broth culture of each strain was mixed with the plasma, and the tubes were incubated at 37 C. for four hours. Any degree of coagulation was interpreted as a positive reaction. For mannitol fermentation, 1 drop of the broth culture was added to duplicate tubes of a 1% solution of the carbohydrate. Results were read after three days of incubation at 37 C.

Results

Of the 101 strains studied, 5 were coagulase-negative. Most of the coagulase-positive strains coagulated plasma in less than two hours of incubation. Six strains could not utilize mannitol as a growth substrate. Four of these mannitol-negative strains were also coagulase-negative. The remaining two mannitol-negative strains were coagulase-positive, but only one was sensitive to the typing phages (groups I and III).

Eighty-eight of the staphylococcic strains were inhibited by less than 1 unit of penicillin per milliliter of test medium. Only two strains could grow in concentrations of penicillin as high as 25 units per milliliter. The minimum inhibitory concentrations (MIC) of penicillin for our culture collection and the distribution of the cultures on the basis of their broad phage-typing groups are shown (Table 1).

Bacteriophage typing showed 31% of the 101 strains to be insensitive to the 25 typing phages. Of the 70 typeable strains, 19 (27%) were type 42D (group IV), and 29 (41%) were multigroup types. Nine strains (13%) were typed as group III and an equal number as miscellaneous (44A). Three strains belonged to group I, and a single strain was typed as group II.

Of the 52 cultures studied for *alpha* and *beta* toxin formation, 23 (44%) produced no demonstrable *beta* toxin. All of the cultures tested produced some *alpha* toxin. The average *alpha* toxin titer for the 30 typeable strains was 1:340, and the average *beta* titer was 1:156. The titer ranges for *alpha* and *beta* toxins were 1:40 to 1:1,280 and zero to 1:1,280, respectively.

TABLE 1—Penicillin Sensitivities of Staphylococcic Strains

Phage type group[s]	MIC of penicillin (units/cc. of medium)				Totals by typing groups
	<1	1–5	5–10	10–25	
I	3	..	..	..	3
II	1	..	..	..	1
III	4	3	2	..	9
IV	18	1	..	..	19
Misc.	8	..	1	..	9
I, III	3	..	..	..	3
I, II, III	4	..	..	..	4
I, III, IV	1	..	..	..	1
II, III	2	..	..	..	2
III, IV	10	1	..	..	11
I, IV, Misc.	1	..	..	..	1
III, Misc.	3	..	..	..	3
IV, Misc.	1	..	..	..	1
III, IV, Misc.	1	..	..	..	1
I, III, IV, Misc.	2	..	..	..	2
NT	26	2	1	2	31
Totals by MIC values	88	7	4	2	....

MIC = minimum inhibitory concentration. Misc. = miscellaneous group. NT = not typeable.

The results of our toxigenicity studies are summarized (Table 2).

Discussion

At least four of the five coagulase-negative strains found in our culture collection were probably not pathogenic strains. None of the coagulase-negative strains were typeable, and four of them could not ferment mannitol. It would have been useful in this investigation to know which of the 101 strains were originally from animals with acute cases of mastitis and which were from animals with chronic cases. Reid and Wilson [8] found a coagulase-negative strain in a cow with chronic mastitis. All of their isolates from cattle with acute cases of mastitis were shown to be coagulase-positive.

The ability of staphylococci to utilize mannitol in their metabolism should no longer be considered a characteristic feature of pathogenic strains. Smith [10] found that only five of 110 pathogenic strains from various animal sources were negative in mannitol fermentation tests. Of 63 nonpathogenic strains, however, 54 gave positive mannitol results. Reid and Wilson [8] reported similar results. They found that approximately 93% of their isolates from diseased udders and 73% from normal udders fermented mannitol.

Histories of antibiotic therapy in the cows from which our cultures were isolated were not known, but it could be safely assumed that some had been treated previously. In view of the increasing difficulty in controlling staphylococcic mastitis by the use of penicillin, it was surprising to notice the high sensitivity of our cultures to this antibiotic. Our results agreed generally with those obtained by Edwards and Rippon.[4] They found 14 of 120 strains to be resistant to concentrations of penicillin between 10 and 200 μg. per milliliter of test medium. The remainder of their cultures were sensitive to 0.125 μg. per milliliter. Eight of our typeable cultures and five nontypeable required 1 to 25 units of penicillin per milliliter for inhibition. Eighty-eight cultures were inhibited by less than 1 unit of penicillin per milliliter of test medium. All 14 of the resistant strains studied by Edwards and Rippon [4] were group III cultures. It can be seen in our results (Table 1) that five of the eight typeable cultures which required as much as 10 units of penicillin for inhibition were also group III strains.

To say that *beta* toxin production is a characteristic feature of pathogenic staphylococci from animals is misleading, because it suggests exclusiveness. *Beta* toxin may be more commonly associated with animal isolates than human isolates, but by no means is it the only toxin produced. More accurately, it appears that most animal isolates produce both *alpha* and *beta* toxins. Minett [7] reported in 1937 that the proportion of udder strains which formed only *alpha* toxin was comparatively small, and that the great majority produced both *alpha* and *beta* toxins. Only occasionally did he find strains which produced *beta* toxin alone. Our results from a comparatively small number of isolates are partially in agreement with the observations of Minett. Twenty-nine of our cultures produced both toxins, and none produced *beta* toxin alone. Twenty-three cultures, however, produced only *alpha* toxin. Many investigators [1,4,5,8-10] have reported a preponderance of *alpha* and *beta* (or *alpha*, *beta*, and *delta*) toxin-producing staphylococci among cattle isolates, but most of these workers have found *beta* toxin-producing strains to be more prevalent than strains which produced only *alpha* toxin.

The majority of our cultures which produced only *alpha* toxin belonged to the nontypeable group, whereas more group IV cultures produced both toxins than any other typing group. These results, however, could not be correlated with the

TABLE 2—Toxin Production by Staphylococcic Isolates

Typing group[s]	No. tested	Average titer		Titer range	
		alpha	*beta*	*alpha*	*beta*
I	1	1:160	1:40	.....	.....
II	0	.....	.....	.....	.....
III	5	1:544	1:40	1:160–1:640	0–1:160
IV	13	1:307	1:220	1:40 –1:640	0–1:960
M	6	1:293	1:297	1:80 –1:640	0–1:1,280
C	13	1:398	1:182	1:40 –1:1,280	0–1:1,280
NT	14	1:131	1:43	1:20 –1:320	0–1:160

M = miscellaneous group. C = multigroup combinations. NT = not typeable.

quantities of toxin produced by strains of the various typing groups. Nontypeable strains produced less *alpha* toxin than any group of typeable strains (Table 2). Group III strains and certain multigroup strains appeared to be the best *alpha* toxin producers. The miscellaneous strains (44A) produced the most *beta* toxin, whereas group IV (42D) and miscellaneous strains produced fair amounts of both toxins.

Typing staphylococci of animal origin with human typing phages has certain disadvantages. Barnum and Fuller [1] suggested that more critical results might be obtained by typing bovine isolates with phages derived from strains of staphylococci associated with mastitis. Coles [3] has recently reported on the use of "adapted" phages for typing animal isolates. The bacterial host range of human typing phages was altered ". . . by the simple expedience of propagating [them] on animal strains of *Staphylococcus aureus* . . ." Approximately 19% of Coles' isolates could not be typed by his eight adapted phages, but nearly 50% were insensitive to the 24 human phages which he used. The convenience of typing with a minimum number of phages is obvious, but the most encouraging aspect of these results is the greater number of isolates found to be typeable by the adapted phages.

It was pointed out earlier that immunizing agents would have more universal usefulness if they were formulated from strains representing several of the broad phage-typing groups. A rather limited number of specific phage types have been demonstrated by many investigators to represent a large majority of the staphylococcic isolates from animals with bovine mastitis. Types 42D (group IV) and 44A (miscellaneous group) are common phage types found in the United States and other countries of the world. Only 19 of our 70 typeable strains were type 42D, but an additional 17 strains which belonged to the multigroup types were also sensitive to the 42D phage. The most common multigroup combination was III, IV; among these strains, group III was most frequently represented by phage type 81.

References

[1] Barnum, D. A., and Fuller, D. S.: Bacteriophage Typing of the Staphylococci Associated with Bovine Mastitis. Canad. J. Comp. Med., 20, (1956): 173–184.

[2] Blair, J. E., and Carr, M.: The Bacteriophage Typing of Staphylococci. J. Infect. Dis., 93, (1953): 1–13.

[3] Coles, E. H., and Eisenstark, A.: Staphylococcic Phages: II. The Use of Human Typing Phages and Adapted Human Typing Phages in the Typing of *Staphylococcus aureus* of Animal Origin. Am. J. Vet. Res., 20, (Sept., 1959): 835–837.

[4] Edwards, S. J., and Rippon, J. E.: The Characters and Distribution of Certain Staphylococci Pathogenic for the Bovine Udder. J. Comp. Path. & Therap., 67, (1957): 111–125.

[5] Elek, S. D., and Levy, E.: Distribution of Haemolysins in Pathogenic and Nonpathogenic Staphylococci. J. Path. & Bact., 62, (1950): 541–554.

[6] Fleming, L. W.: Studies on Bovine Staphylococcal Mastitis: I. Formulation of Immunizing Agents. Vet. Med., 55, (1960): 72-76.

[7] Minett, F. C.: Studies on Bovine Mastitis: XII. Mastitis Due to Staphylococci. J. Comp. Path. & Therap., 50, (1937): 101–121.

[8] Reid, W. B., and Wilson, J. B.: A Study of the Staphylococci Associated with the Bovine Udder. Am. J. Vet. Res., 20, (1959): 825–831.

[9] Slanetz, L. W., and Bartley, C. H.: The Diagnosis of Staphylococcal Mastitis, with Special Reference to the Characteristics of Mastitis Staphylococci. J. Inf. Dis., 92, (1953): 139–151.

[10] Smith, H. W.: The Examination of Staphylococci of Animal Origin with Particular Regard to the Determination of Criteria of Pathogenicity. J. Comp. Path., 57, (1947): 98–115.

SUMMARIO IN INTERLINGUA

Studios in Bovin Mastitis Staphylococcal. II. Characterisation de Isolatos

Esseva studiate 101 racias de staphylococcos ab mastitis bovin. Omnes, con solmente cinque exceptiones, esseva coagulase-positive, e solmente sex non fermentava mannitol. Sextanta-novem pro cento del isolatos esseva sensibile a 25 phagos de typation—individualmente o in varie combinationes. Le plus grande gruppo de phagotypia consisteva de 19 culturas sensibile pro phago 42D (gruppo IV). Vinti-novem culturas esseva lysate per phagos de duo o plus del grande gruppos de phagotypia.

Esseva trovate que 88 del 101 racias esseva sensibile pro minus que 1 unitate de penicillina, secundo le methodo a dilution in tubos. Solmente duo racias requireva le maximo de 25 unitates pro lor inhibition.

Omne le 52 racias examinate pro lor production de toxina alpha e beta produceva un certe

quantitate de toxina alpha. Quaranta-quatro pro cento produceva nulle demonstrabile toxina beta. Racias de gruppo III e racias multigruppal produceva le plus grande quantitates de toxina alpha, durante que le racias miscellanee (phagotypo 44A) pareva esser le melior productores de toxina beta. Racias de gruppo IV (42D) e racias miscellanee produceva significative quantitates de ambe le toxinas. A generalmente parlar, le culturas que poteva esser typate produceva plus alte quantitates de toxina alpha e beta que le culturas que non poteva esser typate.

Quantitative Coagulase Activity and Phage Patterns of Strains of *Staphylococcus aureus* Isolated from Cows in Michigan

John J. Solomon, M.S.; C. L. San Clemente, Ph.D.; A. R. Drury, D.V.M.

SUMMARY

Of 164 staphylococci isolated from bovine udders, 125 were found to be typeable, using the basic set of 24 International-Blair phages and 6 Seto-Wilson phages. The most active phage, S2, lysed 46% of the typeable cultures. Phage patterns S2, S6, and S1/S3 were common to more than one farm, with S2 being the most frequent. Many other patterns were obtained, showing similarity within the same herd. There appeared to be no relationship between cocci isolated from the cervices and udders of cows when compared by phage sensitivity and coagulase production. *Staphylococcus aureus* was not commonly found in the reproductive tracts of repeat breeder cows. Quantitative coagulase production varied widely among the strains isolated from the udder, even among similar phage types. Non-typeable organisms usually were low in coagulase production. Many of the phages of the International-Blair series lysed staphylococci isolated from the udder, but the resulting patterns were different from those isolated from human infections. Staphylococci of two or three different phage types were obtained from the same cow in about 10% of the infected animals having typeable staphylococci. Unlike the so-called epidemic strain, phage type 80/81, reported commonly in human infections, no one widely distributed phage type was found in the bovine population of Michigan.

Received for publication Jan. 3, 1961.

From the Departments of Microbiology & Public Health and Surgery & Medicine, Michigan State University, East Lansing. Published with the permission of the director of the Michigan Agricultural Experiment Station as Journal Article No. 2741.

Some aspects of this work were part of a thesis submitted to the School for Advanced Graduate Studies, Michigan State University, by the senior author in partial fulfillment of the Ph.D. requirements.

This study was supported in part by grant E-2777(RI) from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

The writers thank Dr. J. A. Williams and Dr. D. W. McWade of the College of Veterinary Medicine for assistance in this study.

INTRODUCTION

Staphylococcus aureus has been recognized in recent years as an important organism in the etiology of mastitis. Investigations have revealed that it is present in apparently normal mammary glands [8, 11] and elsewhere in the bovine environment.[4, 17] Staphylococci incriminated in human infections have been found in the bovine udder [9, 18] and external nares,[9] and strains isolated from dairy animals and personnel have the same phage pattern of lysis.[4, 9, 17, 18] Williams Smith [19] found that staphylococci from bovine mastitis, human mastitis, and food poisoning were lysed by the same phage and were antigenically identical. Cultures from all three sources were recoverable from the udder after artificial inoculation. There is evidence, therefore, that staphylococci involved in human infections may be transmitted to animals, or may occur as a result of animal infection. The present investigation was an attempt to determine the relationship, if any, between staphylococci found in bovine udders and the prevailing phage types in human infections in a cross section of the farms of Michigan. A second aim was to determine if a widely distributed phage type was associated with bovine mastitis, as phage type 80/81 has been in human infections.[3] In addition, swab samples were obtained from the reproductive tracts of various "repeat breeder" cows to determine the possible role of staphylococci in reproductive difficulties, and the relationship of these staphylococci to those obtained from the udder. Easley *et al.*[5] reported that uterine infection occurred commonly in repeat breeder cows. The predominant organism was apparently *Staphylococcus aureus*. Gunter and co-workers [6] reported only 5% of uterine and cervical swab samples from repeat breeder cows were sterile, while 33% from normally

breeding cows gave no growth. Coagulase-positive strains of *Staphylococcus aureus* were isolated from 30% of 20 cultures of micrococci isolated from the reproductive tracts of repeat breeder cows.

Materials and Methods

Milk Samples.—Cows with mastitis or reproductive difficulties, or both, were separated from the herd. Strip cup examination was made of the mammary secretion of each quarter prior to obtaining the sample for bacteriologic examination. The udder was washed with a bactericidal solution. Particular attention was paid to washing the teat orifice. Approximately 10 ml. of milk from each quarter was collected in sterile tubes, incubated at 37 C. for 24 hours, and examined by direct microscopic procedure. The freshly collected milk was streaked on tryptose agar containing 5% bovine blood from selected animals. The plates were incubated for 24 hours at 37 C., and colonies were selected for microscopic observation and coagulase determination.

Reproductive Tract Samples.—The cows were prepared for sampling by scrubbing the region around the anus and vulva with soap and then flushing with a bactericidal solution. A sterile glass speculum was then inserted into the vagina and a sample of material near the cervix was obtained by inserting a sterile swab attached to a long flexible metal rod contained in a stainless steel tube. The swab was placed in nutrient broth and held overnight at 37 C. Both swab and broth were then streaked on Bacto *Staphylococcus* 110 agar (Difco), and the resulting colonies were selected for further study.

Phage Preparation.—A set of 24 International phages was obtained from Blair * and the six Seto-Wilson phages were obtained from Wilson.** The method used for phage propagation was the soft agar layer procedure recommended by the Communicable Disease Center, USPHS. The final phage preparation was obtained by freezing and thawing the agar containing the phage-staphylococcic mixture. The syneretic fluid was withdrawn by pipette and assayed by the agar layer method.[1] It was then filtered through a Swinney filter containing approximately one-half thickness of a Seitz filter disc † and again assayed. The critical test dilution (CTD) was used to determine the lytic pattern of the staphylococci, and was the highest tenfold dilution ($\leq 10^{-8}$) which caused confluent lysis of the propagating strain. It was stored at 4 C.

In some cases, phage was prepared in fluid culture by inoculating flasks of trypticase soy broth with equal volumes of concentrated phage and five- to six-hour culture of propagating strain. The flasks were then incubated for four to six hours at 37 C. on a rotary shaker and the resulting clear or turbid material was assayed for phage activity. Good titers were usually obtained by this method (10^{8} to 10^{11} particles per ml.).

Phage Typing.—One to 2 ml. of a three- to five-hour trypticase soy broth culture of each of the staphylococcic isolates was flooded over the surface of a trypticase soy agar plate previously dried at room temperature. The excess inoculum was removed and plates again were allowed to dry. A drop of phage was then added to a marked area of the plate by means of a one ml. syringe fitted with a 27-gauge needle. Each phage was tested against its own propagating strain during each experiment, and every phage was further tested against all propagating strains when initially prepared or prepared anew. The lytic pattern of the propagating strains when cross-tested with each of the typing phages compared favorably with those from the issuing laboratories.

Quantitative Coagulase Determination.—Quantitative coagulase production was determined by using a tube dilution technique.[16] The isolates were grown for five days at 37 C. in brain-heart infusion broth. The cells were centrifuged and 1 ml. of supernatant fluid was diluted serially with an equal volume of 2% peptone-saline solution containing 1:5,000 thimerosal. One milliliter of dilute plasma (1:5 in physiologic saline solution) was then added to each tube. After thorough mixing, the tubes were incubated in a 37 C. water bath. Results were recorded after four and twenty-four hours of incubation and were expressed as the reciprocal of the dilution of the supernatant fluid. The end point, obtained after 24 hours, was the last tube with a one plus reaction. Tubes were graded according to the degree of firmness of the clot. Tubes in which the entire contents had coagulated were labeled as four plus, and those which contained a large, organized clot as three plus. A small, organized clot was indicated by a two plus reading, and small, unorganized clots as one plus.

Plasma was obtained from outdated citrated human blood. Each plasma sample, diluted 1:5 with saline solution, was first tested with coagulase from a known coagulase-producing *Staphylococcus*. Complementary plasma samples were then pooled, dispensed in 10-ml. amounts, and frozen.

Results and Discussion

Milk and cervical samples were obtained from 124 cows located on 23 farms in the lower peninsula of Michigan. Of the 496 quarters sampled, 164 (from 93 cows) yielded staphylococci which were phage typed (Tables 1–3). Table 1 indicates that there were 42 phage patterns on the 23 farms. Three of the patterns, S2, S6, and S1/S3 (Table 3), were common to more than one farm. One pattern, S2, remained unchanged during two visits to the same farm, leaving 31 different phage patterns on the 23 farms. From these data, it was

* Dr. John E. Blair, Hospital for Joint Diseases, New York, N.Y.

** Dr. J. B. Wilson, University of Wisconsin, Madison.

† No. 423FD, Becton, Dickinson and Co., Rutherford, N.J.

TABLE 1—The Number of Phage Patterns of Strains of Staphylococcus aureus Isolated from the Udders of Selected Cows from 23 Herds

Herd	Cows yielding staphylococci	Quarters yielding staphylococci	No. of phage patterns	No. of organisms not typeable
A	3	6	1	5
B	2	5	1	2
C	2	3	1	0
D	4	10	4	3
E	3	3	1	2
F	3	5	1	0
G	2	3	1	2
H	3	5	2	0
I	4	12	1	2
J	2	4	2	2
K	4	11	2	3
L	1	1	1	0
M	3	6	1	1
N	2	2	2	0
O	4	5	1	0
P	9	16	4	5
Q	8	8	2	1
Q (2nd visit)	17	32	3	8
R	4	9	4	0
S	6	10	3	0
T	3	3	2	1
U	1	1	0	1
V	2	3	2	0
W	1	1	0	1
Totals	93	164	42*	39

* Of these 42 phage patterns, only 31 were different from each other.

apparent that the phage types of staphylococci found on the farms in Michigan were quite varied, and that there was no single widely distributed phage type as reported in human infections. None of the patterns prevalent in human infections was observed (80/81, 80/81/52/52A, 7, 7/77, 3A/3B/3C/55, and 52A/79 *); however, 6 organisms from two farms gave a 52/52A/80 pattern. Eleven organisms from five different farms gave wide patterns of lysis, being acted on by 12 to 20 of the 30 phages used. In addition, a large number of lytic reactions (58% of the total) was due to the International Blair phages, indicating a possible cross-infectivity or colonization between man and animal.

The most active of the 30 phages (Table 2) was S2 which caused lysis in 57 (45.6%) of the 125 sensitive strains. This finding was in agreement with that of Seto and Wilson,[14] who found that S2 was the predominant type in their study, lysing 49% of the sensitive cultures tested. It was also our most prevalent type (Table 3).

Many workers [2, 4, 19] have found 42D as the predominant type among bovine staphylococci. It should be recalled that both phages S2 and S1 were derived originally

* Dr. Stanley Read, Michigan Department of Health, Lansing: Personal communication, 1960.

TABLE 2—Frequency of Lysis by the Most Active Phages Upon 125 Sensitive Strains of Staphylococci

Phage	No. of strains lysed	Per cent of strains lysed
S2	57	45.6
S1	45	36.0
S3	44	35.2
42E	36	28.8
42B	35	28.0
S6	31	24.8
44A	29	23.2
S5	28	22.4
54	22	17.6
S4	21	16.8
81	20	16.0

Most strains were lysed by more than one phage.

TABLE 3—Frequency of the Most Common Phage Patterns Among Bovine Staphylococci

Herd	Phage pattern and No. of organisms		
	S2	S6	S1/S3
B	0	3	0
C	0	0	3
D	2	0	0
E	0	1	0
H	4	0	0
I	0	0	10
J	0	1	0
K	0	0	2
N	2	0	0
P	0	3	3
Q (1st visit)	3	0	0
Q (2nd visit)	13	0	0
T	0	1	0
Totals	24	9	18

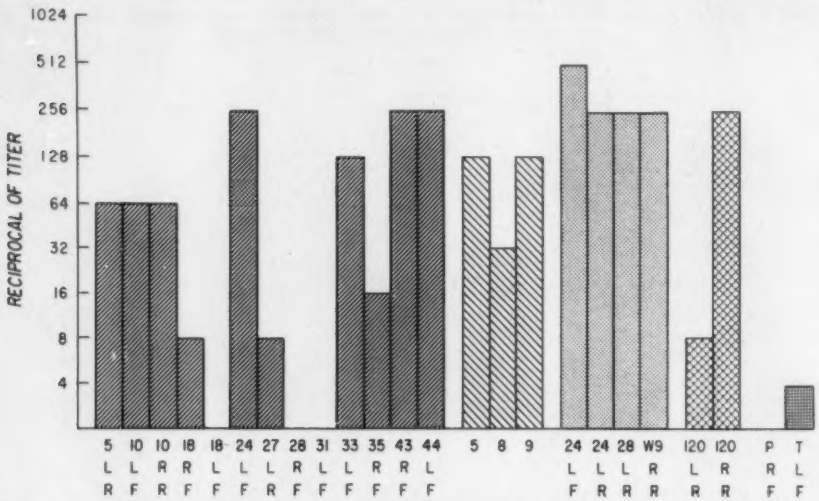

RECIPROCAL OF TITER
1024
512
256
128
64
32
16
8
4
5 10 10 18 18 24 27 28 31 33 35 43 44 5 8 9 24 24 28 W9 120 120 P T
L L R R L L L R L L R R L L R L L L R R L R R R
R F R F F F R F F F F F F F F F R R R R R R F F

Fig. 1—Quantitative production of coagulase by S2 type staphylococci isolated from four herds (bargraphs) and identified by cow number and quarter (abscissa).

from 42D by propagation in different strains of staphylococci of bovine origin.[13] In our experience, however, phage 42D was not very active against the bovine staphylococci. Phages 42B, 42E, and 44A were the most active of the human series of typing phages (Table 2) and frequently all lysed the same clinical isolate. Rountree[12] reported that phages 42B and 42E lysed staphylococci of human origin, while 44A attacked both those of human and bovine origin.

Individual cows were found to carry a variety of strains of staphylococci. While it was theoretically possible to have a different type from each of the four quarters,

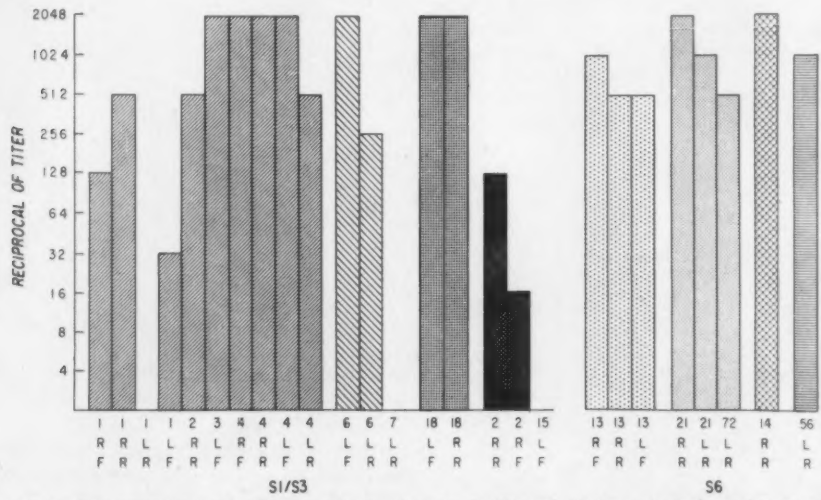

RECIPROCAL OF TITER
2048
1024
512
256
128
64
32
16
8
4
1 1 1 1 2 3 4 4 4 4 6 6 7 18 18 2 2 15 13 13 13 21 21 72 14 56
R R L L R L R R L L L L L L R R R R L R L L L R R R
F R R F R F F R F R F R R F R R F F F R F R R R R R
S1/S3
S6

Fig. 2—The quantitative production of coagulase by S1/S3 and S6 type staphylococci isolated from eight herds (bargraphs) and identified by cow number and quarter (abscissa).

we found 8 animals that carried two types and 1 that harbored three different phage types. We made no attempt in this study to type more than one typical isolate from each quarter. Price *et al.*[10] and Williams Smith[19] have shown that infection is nearly always due to one type of staphylococcus.

Twenty-four per cent of the cultures obtained from milk were not sensitive to the action of the 30 phages used in this study. Seto and Wilson[14] obtained comparable results in their study where 21% of 102 cultures were nontypeable. Coles and Eisenstark[2] reported 49.7% resistant to the action of all human typing phages, but only 18.8% were resistant to the action of eight adapted phages. A much larger percentage (93%) of the organisms isolated from the reproductive tracts of cows in the present study was not typeable. Twenty-four colonies were obtained from *Staphylococcus* 110 agar, and 15 of these were mannitol-fermenting staphylococci, although none gave evidence of coagulase production. Of these, only one was typeable, having a 3A/75/80/S3 pattern. In contrast to the reports of Easley *et al.*[5] and Gunter *et al.*,[6] our failure to find *Staphylococcus aureus* with any frequency in the reproductive tracts of repeat breeder cows would indicate that this organism does not play a major role in the etiology of reproductive failures.

The milk samples from the animal previously mentioned yielded two staphylococci with a 44A/S2/S5 pattern and one nontypeable culture. In all other animals from which milk and cervical samples were

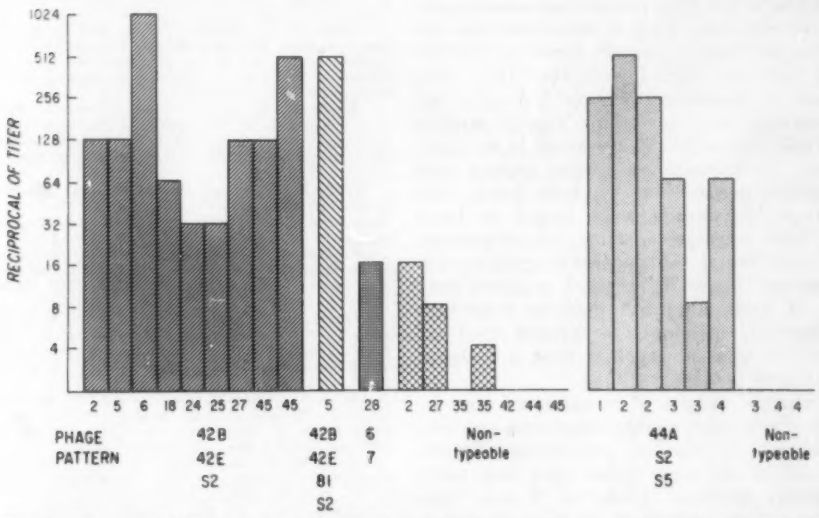


Fig. 3—The quantitative coagulase production and phage patterns of staphylococci isolated from two herds.

positive, the milk samples yielded coagulase-producing cocci, and in all but 3 they were sensitive to one or more of the phages used. On the basis of coagulase production and phage sensitivity, there appears to be little or no relationship between the cocci isolated from the milk samples and those obtained from the cervices.

In order to further characterize the cocci obtained from milk, a quantitative coagulase test was carried out, using 100 of the 164 isolates. The results obtained with some of the more common phage patterns are illustrated (Fig. 1–3). The phage type S2 cocci (Fig. 1) obtained from the four herds (Table 3) were variable in their ability to produce coagulase. Eleven of the 24 organisms with reciprocal titers of 128 or greater produced about the same amount of coagulase even though isolated

from three different farms, while the remaining cocci varied from almost zero to 64. The strains of the S2 phage type varied widely in their production of coagulase. In Figure 2, representing eight herds with phage types S1/S3 and S6, is shown a group of organisms characterized primarily by high coagulase production. Only five exceptions occurred in 26 samples. Cow 4 was interesting in that all of the quarters were infected with an apparently identical strain as defined by phage type and coagulase production. Cow 1, however, was infected in every quarter with the S1/S3 type but each varied from the other with respect to coagulase. Figure 3 depicts the similarities and variations among strains of staphylococci that may occur in an individual herd based on phage typing and coagulase production. In both herds, the nontypeable strains were found to have very low coagulase activity. Consideration of 12 additional nontypeable organisms, not shown in Figure 3, revealed a mixed pattern of high, low, and medium coagulase production, although in almost half of them (5) it was very low, with a reciprocal titer of less than 4.

From the results, it is apparent that in general the nontypeable organisms are uniformly low in coagulase production. Staphylococci of the same phage type may vary markedly in their ability to produce this enzyme. The variability in coagulase production may be the result of strain variation. Smith *et al.*[15] observed high and low coagulase-producing variants within the same strain, and occasionally a variant was found which produced no coagulase. The difference in coagulase production may also be due to an incomplete differentiation of strains by phage typing. Although particular phage patterns are recorded, many of the cultures gave lytic reactions which were too weak to report. It is perhaps desirable to use both the test dilution and concentrated phage when typing organisms in order to give a clear differentiation. Koch and co-workers[7] suggest the use of concentrated phage (1:10 dilution) to avoid possible loss of important phage types.

References

[1] Adams, M. H.: Methods of Study of Bacterial Viruses. In *Methods in Medical Research*, Vol. 2. Year Book Publishers Inc., Chicago, 1950.

[2] Coles, E. H., and Eisenstark, A.: Staphylococcal Phages. II. The Use of Human Typing Phages and "Adapted" Human Typing Phages in the Typing of *Staphylococcus aureus* of Animal Origin. Am. J. Vet. Res., 20, (Sept., 1959): 835–837.

[3] Committee on Staphylococcal Phage Typing—Laboratory Section: Statement on Availability and Uses of Staphylococcal Phage Typing. Am. J. Pub. Health, 49, (1959): 1184–1188.

[4] Edwards, S. J., and Rippon, J. R.: The Characters and Distribution of Certain Staphylococci Pathogenic for the Bovine Udder. J. Comp. Path. & Therap., 67, (1957): 111–125.

[5] Easley, G. T., Leonard, R. H., and Trotter, D. M.: Bacteriological, Pathological, and Clinical Studies of the Reproductive Tract of the Hereford Cow, and A Bacterological Study of Hereford Bull Semen. North Am. Vet., 32, (1951): 258–266.

[6] Gunter, J. J., Collins, W. J., Owen, J., Sorenson, A. M., Scales, J. W., and Alford, J. A.: A Survey of the Bacteria in the Reproductive Tract of Dairy Animals and Their Relationship to Infertility. Am. J. Vet. Res., (April, 1955): 282–285.

[7] Koch, M. L., Kastensen, L. M., and Resnick, A.: Report on Commonly Occurring Phage Patterns in a Hospital in Which Staphylococcal Infections are Endemic. Antibiotics & Chemother., 7, (1959): 409–415.

[8] Kraffenhoft, K. L., Adams, A. P., and Schipper, I. A.: Bacteriological Differentiation of Organisms Isolated from Mastitic and Non-Mastitic Secretions. Vet. Med., 53, (1958): 625–631.

[9] Pagano, J. S., Farrer, S. M., Plotkin, S. A., Brachman, P. S., Fekety, R. F., and Pidcoe, V.: Isolation from Animals of Human Strains of Staphylococci During an Epidemic in a Veterinary School. Science, 131, (1960): 927–928.

[10] Price, P., Neave, F. K., Rippon, L. E., and Williams, R. E. O.: The Use of Phage Typing and Penicillin Sensitivity Tests in Studies of Staphylococci from Bovine Mastitis. J. Dai. Res., 21, (1954): 342–353.

[11] Reid, W. B., and Wilson, J. B.: A Study of the Staphylococci Associated with the Bovine Udder. Am. J. Vet. Res., 20, (Sept., 1959): 825–831.

[12] Rountree, P. M.: The Serological Differentiation of Staphylococcal Bacteriophages. J. Gen. Microbiol., 3, (1949): 164–173.

[13] Seto, J. T., Kaesburg, P., and Wilson, J. B.: Electron Microscopy and Serology of Staphylococcus Phages. J. Bact., 72, (1956): 847–850.

[14] Seto, J. T., and Wilson, J. B.: Bacteriophage Typing of Micrococci of Bovine Origin. Am. J. Vet. Res., 19, (Jan., 1958): 241–246.

[15] Smith, D. D., Morrison, R. B., and Lominski, I.: Isolation of Variants of *Staphylococcus aureus* Giving High and Low Yields of Coagulase. J. Path. & Bact., 64, (1952): 567–573.

[16] Tager, M., and Hales, H. B.: Quantitative Coagulase and Toxin Production by Staphylococci in Relation to the Clinical Source of the Organisms. Yale J. Biol. & Med., 20, (1948): 41–49.

[17] Thorne, H., and Wallmark, G.: Studies on Staphylococcal Mastitis in an Individual Herd with Special Reference to Phage Types of Isolated Staphylococci. Acta Vet. Scand., 1, (1960): 114–119.

[18] Wallace, G. D., Quisenberry, W. B., and DeHarne, H. A.: Preliminary Report of Human Staphylococcal Infection Associated with Mastitis in Dairy Cattle. Pub. Health Rep., 75, (1960): 457–460.

[19] Williams Smith H.: The Typing of Staphylococci of Animal Origin by the Bacteriophage Method. J. Comp. Path. & Therap., 58, (1948): 179–188.

SUMMARIO IN INTERLINGUA

Le Nivellos del Activitate de Coagulase e le Configurationes de Phagotypia in Racias de *Staphylococcus aureus*, Isolate ab Vaccas in Michigan

Inter 164 isolatos staphylococcic ab uberes bovin, 125 se provava typabile intra le serie basic de 24 phagos Blair-International e 6 phagos Seto-Wilson. Le phago le plus active—i.e. S2—lysava 46% del 125 typabile culturas. Le configurationes phagic S2, S6, e S1/S3 esseva commun a plus que un ferma. S2 esseva le plus frequente del tres. Multe altere configurationes esseva obtenite, reflectente similitudes intra le greges individual. Nulle relation esseva apparente inter coccos isolate ab le cervices uterin e le uberes del vaccas quando illos esseva comparate in lor sensibilitate phagic e lor production de coagulase. *Staphylococcus aureus* non esseva trovate communmente in le vias reproductori de vaccas pluriparas. Le nivello del production de coagulase variava extensemente inter le racias isolate ab le ubere, mesmo in le presentia de similitudes phagotypic. Le non-typabile organismos manifestava usualmente un basse grado de production de coagulase. Multes del phagos in le serie Blair-International lysava staphylococcos isolate ab le uberes, sed le resultante configurationes differeva ab illos de isolatos ab infectiones human. Staphylococcos de 2 o 3 differente phagotypos esseva obtenite ab le mesme vacca in circa 10% del casos de animales portante typabile organismos. Nulle phagotypo individual esseva trovate in le population bovin de Michigan con le extense distribution reportate communmente in infectiones human pro le si-appellate racia epidemic, i.e. le phagotypo 80/81.

The Comparative Susceptibility of Calves and Adult Cattle to Bovine Anaplasmosis

T. O. Roby, D.V.M., M.S.; D. W. Gates, V.M.D., M.S.; L. O. Mott, D.V.M.

SUMMARY

Comparative infectivity titrations on *Anaplasma*-infected blood were conducted in splenectomized and nonsplenectomized calves and cows. The results indicate that the most susceptible animal was the splenectomized cow. Splenectomized calves and nonsplenectomized cows were found to have a similar susceptibility to *Anaplasma*-infected blood. The nonsplenectomized calf was the least susceptible animal.

INTRODUCTION

The effect of removal of the spleen (splenectomy) from cattle which have anaplasmosis was first reported in 1926 by deKock and Quinlan.[1] They described the clinical relapse in infected carrier cattle following splenectomy. Subsequently, other workers found that splenectomy of noninfected cattle resulted in increased susceptibility to the disease.[3, 8, 13]

Anaplasmosis research studies frequently involve splenectomized calves. Calves are easier to obtain, less expensive to maintain, and more convenient to work with than adult cattle. However, most clinical cases of anaplasmosis are seen in adult cattle under field conditions. In this study, we compared the susceptibility to anaplasmosis of calves and cows, both normal and splenectomized, when inoculated with carrier blood. The comparion was made by determining the minimum infective dose of blood in each type of test animal.

Materials and Methods

An infectivity titration was performed on four groups of cattle consisting of 5 animals per group. The groups of animals were: (1) calves, nonsplenectomized, (2) calves, splenectomized, (3) cows, nonsplenectomized, and (4) cows, splenectomized. The animals were obtained from the anaplasmosis-free herd at Beltsville. Ten cows and 10 male castrated calves of the Holstein-Friesian breed were used. Five of the adults and 5 of the calves were splenectomized[5] 45 to 90 days before inoculation. Three of these animals had eperythrozoonosis[10] for a short time after the operation. These infections were mild, disappeared spontaneously, and were not observed in blood films made after the animals were inoculated with the diluted *Anaplasma*-infected carrier blood. All animals were free from external parasites. The cows ranged in age from 3½ to 11 years and in weight from 1,350 to 1,690 lb. The calves ranged in age from 4½ to 7 months and in weight from 323 to 414 lb.

Test animals of each group were inoculated intravenously with diluted blood from a carrier of *Anaplasma marginale*, animal 2319. Whole blood was collected aseptically into a flask containing sufficient dried sodium citrate to make a final concentration of 0.5%. Before inoculation, the citrated blood was diluted in tenfold serial dilutions in sterile normal bovine serum.

Cow 2319 had been a known carrier of bovine anaplasmosis for 19 years. She had been isolated in screened facilities during her entire lifetime and had never been experimentally re-exposed to any infectious agent. At the time her blood was collected for this study, the anaplasmosis complement-fixation antibody titer of the serum was 4+ at a 1:8 dilution.

A preliminary test in splenectomized calves determined the approximate infective range of the donor's blood. A 10^{-1} dilution of 1.0 ml. was found to be infective. Earlier work[6] had shown that blood of another anaplasmosis carrier, having a complement-fixation serum titer of 1:10, was not infective beyond a dose of 1.0 ml. of a 10^{-1} dilution when inoculated into splenectomized calves. With this background data for predicting the infectivity range, it was postulated that a 1.0-ml. dose of diluted blood from carrier 2319 would become noninfective beyond the 10^{-1} and 10^{-2} dilutions. The dilutions used for each group of animals in the current study are shown (Table 1). The animals were inoculated intravenously with 1.0-ml. doses. The nonsplenectomized calves were started with 1.0 ml. of undiluted blood, since they were expected to be the least susceptible. The splenectomized cows were started at 10^{-2} dilution, since they were expected to be the most susceptible. The blood was diluted immediately before inoculation. Those animals given the highest dilutions were inoculated first. Seventy-three minutes elapsed between the

Received for publication July 27, 1960.

From the National Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, Beltsville, Md. Dr. Mott is now located at the National Animal Disease Laboratory, Ames, Iowa.

The authors thank Mr. P. A. Madden and Mr. Herbert Rogers for their technical assistance.

blood collection and the inoculations into all animals.

The inoculated animals were observed for 90 days. Rectal temperatures were recorded twice daily during this period. Blood specimens were collected three times weekly. Giemsa-stained blood films from each sample were examined microscopically, and the packed cell volume was determined on each sampling. Phenolized serum samples on all blood specimens were tested by the standardized anaplasmosis complement-fixation (CF) method.[11] Infection was indicated by the appearance of *Anaplasma* bodies, clinical signs of anemia, and positive CF reactions. To determine if inapparent infection had occurred in animals not showing signs of anaplasmosis, 1 liter of blood from the first animal negative to anaplasmosis in each titration group was transferred into other susceptible splenectomized calves. The susceptibility of all animals that did not develop anaplasmosis within the 90-day observation period was subsequently tested by inoculation with large doses of blood obtained from cattle in the acute stage of infection with the same isolate of *A. marginale*.

Results

The minimum dilutions of blood from anaplasmosis carrier 2319 that produced infection in each test group are shown in Table 1. Anaplasmosis developed in a nonsplenectomized calf when 1.0 ml. of undiluted blood was inoculated, but not in similar calves when dilutions of 10^{-1} or higher were used. In the splenectomized calves and nonsplenectomized cows, infection developed in those given 1.0 ml. of a 10^{-1} dilution but not in those given higher dilutions. In the splenectomized cows, infection developed when 1.0 ml. of a 10^{-2} dilution was injected, but not with higher dilutions. The animals that remained negative to anaplasmosis were later proved to be susceptible to the disease when their susceptibility was tested. Attempts to demonstrate inapparent infection in 4 animals negative to anaplasmosis were not successful when 1 liter of their blood was inoculated into other splenectomized calves. No other blood infections were observed in any animals after inoculation.

In those animals developing infection, the incubation periods between inoculation and the first appearance of *Anaplasma* bodies were 29 days in 1 animal and 31 days in 3. Positive CF reactions first occurred near the time the *Anaplasma* bodies appeared. Elevations in body temperature occurred during the development of anemia in the 4 infected animals.

Clinical observations on the infected animals are shown (Fig. 1). The disease ran a mild course in the nonsplenectomized calf that developed anaplasmosis. The parasitized erythrocytes did not exceed 10%, and moderate anemia developed. The infection in the nonsplenectomized cow also ran a mild course similar to that seen in the nonsplenectomized calf. The splenectomized calf had a more severe infection; 40% of its erythrocytes were parasitized and it had marked anemia. In all 3 animals, the clini-

TABLE 1—Infectivity Titrations of Anaplasma-Infected Blood Inoculated into Nonsplenectomized and Splenectomized Calves and Cows: Minimum Infective Dilutions and Incubation Periods

Dilutions of blood (intravenous 1.0-ml. dose)	Calves		Cows	
	Nonsplenectomized	Splenectomized	Nonsplenectomized	Splenectomized
	Infectivity titrations			
10^{-0}	+(1.0 ml.)	...	...	...
10^{-1}	—	+(0.1 ml.)	+(0.1 ml.)	...
10^{-2}	—	—	—	+(0.01 ml.)
10^{-3}	—	—	—	—
10^{-4}	—	—	—	—
10^{-5}	...	—	—	—
10^{-6}	...	...	...	—
Postinoculation time when:	Incubation periods in animals developing anaplasmosis (days)			
Anaplasma bodies appeared	29	31	31	31
Positive CF reaction appeared	27	33	28	33
Temperature first elevated above 104 F.	31	37	37	35

+ = Positive reaction (actual quantity of carrier blood producing anaplasmosis is shown in parentheses); — = negative reaction (all negative animals were susceptible at challenge 90 to 120 days after inoculation).

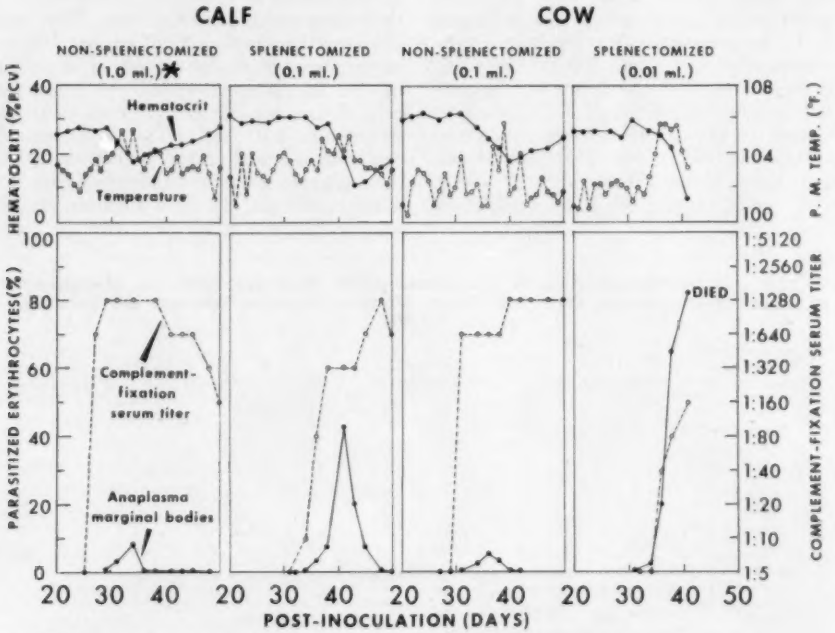
cal signs disappeared spontaneously, but the animals remained carriers and had no evidence of infection other than a persistent CF serum titer. The splenectomized cow that became infected developed severe clinical signs of anaplasmosis and died ten days after the *Anaplasma* bodies first appeared. On the day before death, 83% of its erythrocytes were parasitized with *Anaplasma* bodies. The CF antibody titers increased rapidly in all 4 animals near the time *Anaplasma* bodies first appeared and then decreased gradually in the 3 survivors.

Discussion

The determination of exact infectivity endpoints would require more than 1 test animal per dilution of blood. However, the method employed demonstrated differences between the susceptibility of splenectomized and nonsplenectomized cows and calves to anaplasmosis infection. Calves with intact spleens required the largest amount of blood for infection, whereas splenectomized cows required much less blood for infection. The same doses of blood infected both calves without spleens and cows with spleens. These findings agree with the concept that susceptibility to anaplasmosis increases upon maturity and splenectomy. Furthermore, they suggest a similar degree of susceptibility between splenectomized calves and nonsplenectomized adult cattle.

The dose of blood producing infection was smallest when inoculated into splenectomized cows. The marked susceptibility of splenectomized cattle to anaplasmosis was also illustrated in the report of Quinlan *et al.*,[12] when an animal infected with the "mild" *Anaplasma centrale* strain reacted so severely after splenectomy that it died.

Comparable quantities of diluted blood (1.0 ml. of a 10^{-1} dilution) represented a minimum infective dose for the splenectomized calf and nonsplenectomized cows. The similarity in susceptibility of these



* The dose or volume of carrier blood inoculated is expressed in milliliters.

Fig. 1—Anaplasmosis produced by minimum infective doses of carrier blood inoculated into nonsplenectomized and splenectomized calves and cows. The percentage of parasitized erythrocytes, complement-fixing antibody serum titers, packed cell volume, and temperature reactions are shown.

two types of animals supports the experimental use of splenectomized calves in studies of the disease. The differences in manifestation of clinical signs resulting from the same infective dose in the two types of animals may be due to individual variations, as well as to differences in their ages and splenic activity. It has been emphasized by Lotze [7] that the severity of anaplasmosis infection is subject to considerable variation and is related to the quantity and character of the infecting inoculum, as well as to age and individual susceptibility.

While individual animal variation may have influenced the severity of the disease, the small quantities of infective blood used in this study also may have influenced the course of the disease. It has previously been shown that a large number of subclinical cases of anaplasmosis occur in herds of infected cattle.[9] Apparently, transmission of the disease among cows frequently occurs without severe clinical manifestations. It is believed that biting insects are responsible for much of the anaplasmosis transmission in enzootic areas of the southeastern United States, as ticks are rarely found on cattle.[2] Although the quantity and character of the infectious agent spread by such insects are undetermined, it would appear likely that in many instances the infective dose is quite small.

References

[1] deKock, G., and Quinlan, J.: Splenectomy in Domestic Animals and Its Sequelae, with Special Reference to Anaplasmosis in Sheep. Union of South Africa, 11th and 12th Rep., Director of Veterinary Education and Research. Part 1, (Sept., 1926): 369–480.

[2] Dikmans, G.: The Transmission of Anaplasmosis. Am. J. Vet. Res., 11, (Jan., 1950): 5–16.

[3] Dykstra, R. R., Roderick, L. M., Farley, H., McMahan, U. K., and Splitter, E. J.: Studies in Anaplasmosis. II. 1938–48, Kansas State College, Agric. Exper. Sta., Tech. Bull. 66, (1948): 1–21.

[4] Foote, L. E., Levy, H. E., Torbert, B. J., and Oglesby, W. T.: Interference Between Anaplasmosis and Eperythrozoonosis in Splenectomized Cattle. Am. J. Vet. Res., 18, (July, 1957): 556–559.

[5] Gates, D. W.: The Technique Employed in Splenectomy of Bovine Animals. J.A.V.M.A., 122, (Feb., 1953): 94–98.

[6] Gates, D. W., Madden, P. A., Martin, W. H., and Roby, T. O.: The Infectivity of Blood from *Anaplasma*-Infected Cattle as Shown by Calf Inoculation. Am. J. Vet. Res., 18, (April, 1957): 257–260.

[7] Lotze, J. C.: Variables and Constants in Experimental Bovine Anaplasmosis and Their Relationship to Chemotherapy. Am. J. Vet. Res., 8, (July, 1947): 267–274.

[8] Mohler, J. R.: Report of the Chief, Bureau of Animal Industry, U.S. Department of Agriculture, (1937): 46–47.

[9] Mott, L. O., Saulmon, E. E., Roby, T. O., and Martin, W. H.: Field Control Studies on Anaplasmosis. Proc. U.S. Livestock San. A. (1959): 68–73.

[10] Neitz, W. O.: Eperythrozoonosis in Cattle. Onderstepoort J. Vet. Sci. and Anim. Indust., 14, (1940): 9–24.

[11] Peterson, K. J., Christensen, J., Henderson, J. A., King, J. A., Oglesby, W. T., Poelma, L. J., Roby, T. O., and Willers, E. H.: Report of the Committee on Anaplasmosis. Proc. U.S. Livestock San. A. ((1957): 79–92

[12] Quinlan, J., deKock, G., and Marais, I. P.: The Operation of Splenectomy in Horses, Cattle, Sheep, Goats, Pigs, Dogs, and Some South African Antelopes. A Summary of the Results of 98 Splenectomies. Onderstepoort J. Vet. Sci. and Anim. Indust., 5, (1935): 273–303.

[13] Simpson, C. F., and Saunders, D. A.: Diagnosis of the Carrier Stage of Anaplasmosis Under Experimental Conditions. Vet. Med., 48, (1953): 182–184.

SUMMARIO IN INTERLINGUA

Le Susceptibilitate Comparative de Vitellos e Bestial Adulte pro Anaplasmosis Bovin

Comparative titrationes de infectivate con sanguine inficite per *Anaplasma* esseva conducite in splenectomisate e non-splenectomisate vitellos e vaccas. Le resultatos indica que le animal le plus susceptibile esseva le splenectomisate vacca. Esseva constatate que splenectomisate vitellos e non-splenectomisate vaccas habeva un simile susceptibilitate pro sanguine inficite de *Anaplasma*. Le non-splenectomisate vitello esseva le animal le minus susceptibile.

Polysaccharide and Protein Relationships of Normal Bovine Serum

George T. Dimopoullos, Ph.D.

SUMMARY

In a study of the relationship of total protein-bound polysaccharide and total serum protein of normal bovine serum, analyses were made on the serum of 215 female cattle ranging in age from less than 3 months to more than 84 months. Young animals possessed less serum-bound polysaccharide and serum protein than the older animals, and the ratios of polysaccharide to protein remained essentially similar regardless of age.

INTRODUCTION

There is still little information on the origin and physiologic significance of the serum-bound polysaccharides.[25] There has been considerable speculation on this subject, and workers have advanced the theory that serum-bound polysaccharides arise during tissue proliferative processes when rapid cell growth and protein synthesis are taking place.[15] Others have stated that, in protein catabolism, these polysaccharides originate during the destruction of tissues or as a result of the depolymerization of connective tissue ground substance.[2,9,14] In any case, the concentration of these serum-bound polysaccharides is increased in infectious [7,14,19,22-24] and neoplastic diseases,[2,6,11,14] injury and inflammation,[1,15] stress,[1,9] and rheumatic disorders.[8,12,18,20]

Some attention has been given to the relationship of polysaccharide and its carrier, the serum protein, of normal human serum. When expressed as the polysaccharide:protein ratio, it has been of value as a clinical diagnostic tool in neoplastic [6,11] and rheumatic diseases in man.[12,18,20] In normal human serum, this ratio has been found to increase with age,[5,17] although both polysaccharide and serum protein concentrations tend to increase, also. In separate studies, it was observed that age affected the concentrations of both total serum protein [10,13] and total serum-bound polysaccharide [4] in the bovine species. It appeared that some relationship existed between these two substances in bovine serum. A change in either value would alter the ratio, and these changes might be useful in studying pathogenesis of disease. Since at this laboratory there was interest in these biologic substances as possible aids in determining more clearly some of the pathologic processes involved in infectious diseases, it was necessary to establish normal values for the relationship of polysaccharide to protein and the effect of age on this ratio.

Materials and Methods

A total of 215 female animals of single and mixed dairy breeding were used in this study. Their ages varied from less than 3 months to more than 84 months. All animals were managed under southern Louisiana conditions and were considered to be free from any apparent physiologic or pathologic disturbances. The numbers of animals in each age group are given (Table 1).

Blood was collected by jugular venipuncture and allowed to clot. The serum, obtained by centrifugation and decantation, was stored in sealed vials at −22 C. until it was analyzed.

Total Serum Protein.—The biuret reaction was used in the analyses of all serums.[21] Duplicate samples, in 0.2-ml. amounts, were pipetted into tubes adapted for use as spectrophotometric cuvettes. An 0.85% solution of sodium chloride in 4.8-ml. amounts and 5.0 ml. of biuret reagent were then added. The materials were thoroughly mixed, and the tubes were placed in a water bath at 32 C. for 30 minutes for optimal color development. All tubes were read in a spectrophotometer at 530 mμ. Reference standards and blanks were included in all analyses.

Total Serum-Bound Polysaccharide.—The tryp-

Received for publication March 27, 1961.

From the Department of Veterinary Science, Louisiana Agricultural Experiment Station, Louisiana State University, Baton Rouge.

Supported in part by grant E-2250 from the National Institutes of Health, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

The author thanks Barbara H. Fletcher for assistance in determining total serum-bound polysaccharide.

TABLE 1—Total Serum-Bound Polysaccharide, Total Serum Protein, and Polysaccharide: Protein Ratios of Normal Bovine Serum in Cows of Nine Age Groups

Age (months)	No. of animals	Age group mean values		
		Total serum-bound polysaccharide*	Total serum protein**	Polysaccharide: protein ratio†
>84	38	121.80	8.12	1.50
48–84	33	122.45	8.06	1.52
36–48	8	123.21	7.82	1.58
24–36	30	111.03	7.42	1.50
18–24	27	109.24	7.18	1.52
12–18	26	112.00	6.96	1.61
6–12	22	107.50	6.83	1.57
3–6	20	102.04	6.60	1.55
<3	11	93.13	6.26	1.49

The mean polysaccharide:protein ratio for the 215 animals when considered as a group was 1.53. Correlation coefficient of the relationship of polysaccharide and protein was calculated as + 0.52 for the 215 animals.

* Total serum-bound polysaccharide as milligrams per 100 ml. of serum. ** Total serum protein as grams per 100 ml. of serum. † Grams of polysaccharide/grams of serum protein x 100 = polysaccharide: protein ratio.

tophane reaction of Shetlar *et al.*[16] was used for the determination of total bound polysaccharide. Serums were diluted 1:2 with 0.9% sodium chloride solution, and 0.2-ml. amounts of the mixtures were pipetted dropwise into duplicate sets of spectrophotometric tubes containing 10 ml. of absolute ethanol. The precipitates were centrifuged at 700g for 15 minutes, and the alcohol was decanted. An additional aliquot of 10 ml. of absolute ethanol was added, and the precipitate was centrifuged again. The alcohol was removed, and the precipitates were drained by inversion of the tubes. Distilled water in 2-ml. amounts and 77% sulfuric acid in 7-ml. amounts were then added to each tube, and the contents were allowed to stand at 25 C. for ten minutes to allow the precipitates to dissolve. The tubes were placed in an ice bath at 5 C. for 15 minutes, and 1.0 ml. of a cold aqueous solution of 1.0% l-tryptophan was added to each sample. The contents of each tube were thoroughly mixed and immediately placed in a water bath at 100 C. for 20 minutes. Each tube was shaken once after heating. All tubes were cooled in an ice bath for five minutes and then allowed to stand for 25 minutes at 25 C. for full color development. Optical densities were read at 580 mμ. Reference standards and blanks were included with each group of determinations.

Results and Discussion

Mean group values of total serum-bound polysaccharide, total serum protein, and polysaccharide:protein ratios are shown (Table 1). It was evident that both polysaccharide and serum protein levels increased significantly with an increase in the age of the animals. These facts were substantiated in other reports dealing singly with polysaccharide [4] and serum protein studies.[10,13] Statistical analyses showed the differences in these values between age groups to be highly significant.[4,10] The polysaccharide:protein ratios remained relatively constant, as the data indicate in the present studies, even though the concentrations of polysaccharide and protein increased with an increase in the age of the animals. This ratio did not change significantly, indicating that, at least in normal bovine serum, the polysaccharide and protein were interrelated so that relatively constant ratios were present in animals of all the age groups that were represented.

Statistical analyses of the relationship between total serum-bound polysaccharide and total serum protein gave a correlation coefficient of + 0.52. This value indicated that there was a highly significant correlation between the amounts of polysaccharide and protein in normal bovine serum.

Several workers have independently reported that age influences the amount of serum protein present in human [5,17] and in bovine serums,[10,13] whereas others have shown that polysaccharide concentrations increase with age in bovine,[4] human,[5,17] and rat [1] serums.

The relationship of polysaccharide and protein in bovine serum has not received attention in veterinary research. One report was made on a study of the polysaccharide content in 13 samples of bovine serum obtained from animals of unspecified age.[3] The average concentration of total serum-bound polysaccharide was found to be 124 mg./100 ml. of serum while the polysaccharide:protein ratio was 1.53. The protein content of the serum samples averaged 8.08 Gm./100 ml. of serum.

The ability of normal serum protein to bind polysaccharide, per se, has not been

discussed previously. In the present studies, it was shown that normal serum protein can bind definite amounts of polysaccharide in relatively constant ratios, regardless of the concentration of protein. The ratios in cows of each age group demonstrated this fact since they were similar in magnitude.

In disease, the ability of serum protein to bind polysaccharide apparently increases, thereby increasing the polysaccharide: protein ratio. This increased binding capacity could be due to an alteration in binding sites in serum protein molecules, resulting from changes in molecular groupings formed during the synthesis of antibody or abnormal serum protein components. It should be considered also that, in disease, additional polysaccharide may be bound due to an increase in the concentration of serum protein. This increased ability of serum to bind polysaccharide as the protein concentration increases with age has been demonstrated in this study of normal bovine serum.

References

[1] Boas, N. F., and Peterman, A. F.: Effects of Age, Food Intake, and Stress on Plasma Hexosamine Levels in the Rat. Proc. Soc. Exptl. Biol. & Med., 82, (1953): 19–24.

[2] Catchpole, H. R.: Serum and Tissue Glycoproteins in Mice Bearing Transplantable Tumors. Proc. Soc. Exptl. Biol. & Med., 75, (1950): 221–223.

[3] Decker, B., McKenzie, B. F., and McGuckin, W. F.: Zone Electrophoretic Studies of Proteins and Glycoproteins of Bovine Serum and Synovial Fluid. Proc. Soc. Exptl. Biol. & Med., 102, (1959): 616–618.

[4] Dimopoullos, G. T., Schrader, G. T., and Fletcher, B. H.: Electrophoretic Studies of Bovine Serum. IV. Differences in Serum Glycoproteins Due to Age and Sex. Proc. Soc. Exptl. Biol. & Med., 102, (1959): 704–707.

[5] Good, R. A., Kelley, V. C., Good, T. A., and Glick, D.: Mucolytic Enzyme Systems. XX. Comparison of Mucoprotein and Hyaluronidase Inhibitor Concentrations in Maternal and Infant Serum. Pediatrics, 12, (1953): 575–583.

[6] Greenspan, E. M., Tepper, B., Terry, L. L., and Schoenbach, E. B.: The Serum Mucoproteins as an Aid in the Differentiation of Neoplastic from Primary Parenchymatous Liver Disease. J. Lab. & Clin. Med., 39, (1952): 44–56.

[7] Hudgins, P. C., and Patnode, R. A.: Electrophoretic Distribution of Serum Protein and Glycoprotein in the Tuberculous Rat, Rabbit and Guinea Pig. Proc. Soc. Exptl. Biol. & Med., 95, (1957): 181–183.

[8] Kelley, V. C., Adams, F. H., and Good, R. A.: Serum Mucoproteins in Patients with Rheumatic Fever. Pediatrics, 12, (1953): 607–621.

[9] Kushner, D. S., Honig, K., Dubin, A., Dyniewics, H. A., Bronsky, D., de la Huerga, J., and Popper, H.: Studies of Serum Mucoprotein (Seromucoid). II. Physiologic Variations and Response to Stress. J. Lab. & Clin. Med., 47, (1956): 409–417.

[10] Larson, B. L., and Touchberry, R. W.: Blood Serum Protein Level as a Function of Age. J. Anim. Sci., 18, (1959): 983–990.

[11] Moschides, E., Stefanini, M., Magalini, S. I., and Kistner, S. A.: The "Mucoprotein Fraction" (Phosphotungstic Acid Precipitate) in Tissue and Serum: A Comparison of Normal Findings and Findings in Leukemia and Lymphoma. J. Lab. & Clin. Med., 50, (1957): 216–224.

[12] Payne, R. W., Shetlar, M. R., Bullock, J. A., Patrick, D. R., Heilbaum, A. A., and Ishmael, W. K.: The Serum Polysaccharide Protein Ratio (PR) as a Measure of Rheumatoid Activity. Ann. Int. Med., 41, (1954): 775–779.

[13] Pierce, A. E.: Electrophoretic and Immunological Studies on Sera from Calves from Birth to Weaning. I. Electrophoretic Studies. J. Hyg. (London), 53, (1955): 247–260.

[14] Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W.: Variation in Protein and Polysaccharide Content of Sera in the Chronic Diseases, Tuberculosis, Sarcoidosis, and Carcinoma. J. Clin. Invest., 26, (1947): 90–102.

[15] Shetlar, M. R., Bryan, R. S., Foster, J. V., Shetlar, C. L., and Everett, M. R.: Serum Polysaccharide Levels in Experimental Inflammation. Proc. Soc. Exptl. Biol. & Med., 72, (1949): 294–296.

[16] Shetlar, M. R., Foster, J. V., and Everett, M. R.: Determination of Serum Polysaccharides by the Tryptophane Reaction. Proc. Soc. Exptl. Biol. & Med., 67, (1948): 125–130.

[17] Shetlar, M. R., Foster, J. V., Kelly, K. H., and Everett, M. R.: Serum Polysaccharide Level in the Normal State. Proc. Soc. Exptl. Biol. & Med., 69, (1948): 507–511.

[18] Shetlar, M. R., and Payne, R. W.: Objective Evaluation of Patients with Rheumatic Diseases. IV. Comparison of the Diphenylamine Reaction (DPA) with Serum Glycoprotein and Seromucoid Levels. J. Lab. Clin. Med., 51, (1958): 588–591.

[19] Shetlar, M. R., Shetlar, C. L., Payne, R. W., Neher, G. M., and Swenson, C. B.: Serum Protein and Glycoprotein Alteration in Swine with Experimental Arthritis. Proc. Soc. Exptl. Biol. & Med., 98, (1958): 254–256.

[20] Stidworthy, G., Payne, R. W., Shetlar, C. L., and Shetlar, M. R.: Objective Evaluation of Patients with Rheumatic Diseases. II. Paper Electrophoretic Studies of Serum Glycoprotein and Protein from Patients with Rheumatoid Arthritis. J. Clin. Invest., 36, (1957): 309–313.

[21] Weichselbaum, T. E.: An Accurate and Rapid Method for the Determination of Proteins in Small Amounts of Blood Serum and Plasma. Am. J. Clin. Path., 16, (1946): 40–49.

[22] Weimer, H. E., and Redlich-Moshin, J.: Serum Glycoprotein Concentrations in Experimental Tuberculosis of Guinea Pigs. Am. Rev. Tuberc., 68, (1953): 594–602.

[23] Weimer, H. E., Redlich-Moshin, J., and Nelson, E. L.: Comparative Effects of Infection and of Immunization on Serum Glycoprotein Concentrations. J. Immunol., 74, (1955): 243–248.

[24] Weimer, H. E., Redlich-Moshin, J., Quinn, F. A., and Nelson, E. L.: Serial Determinations of Serum Glycoproteins Following Antigen Injection Into Previously Immunized Animals. J. Immunol., 78, (1957): 1–4.

[25] Winzler, R. J.: Determination of Serum Glycoproteins. In "Methods of Biochemical Analysis," Vol. II. (D. Glick, ed.). Interscience Publishers, Inc., New York, N.Y., (1955): 279–311.

SUMMARIO IN INTERLINGUA

Le Effecto del Etate Super le Proportion Polysaccharido:Proteina in Normal Sero Bovin

In un studio del relation inter le total polysaccharido ligate a proteina e le total proteina seral in normal sero bovin, analyses esseva effectuate in le seros de 215 pecias de bestial feminin de varie etates ab 3 usque ad plus que 84 menses. Juvene animales possedeva minus polysaccharido ligate al sero e minus proteina seral que animales de etates plus avantiate, sed le proportion de polysaccharido a proteina remaneva essentialmente le mesme sin reguardo al etate.

A Technique for the Surgical Removal of the Forestomachs of Calves

F. Sauer, D.V.M., Ph.D., and G. J. Brisson, Ph.D.

SUMMARY

Four 3-week-old calves survived more than three months postoperatively when rumen, reticulum, omasum, and part of abomasum were removed surgically.

At necropsy, there was a perforating gastric ulcer in 1 calf. The cause of death in the other 3 did not appear to be directly related to the surgical procedure.

The usefulness of these calves for experimental work was limited by their failure to survive more than six months postoperatively and by the fact that gastric vagus innervation was destroyed and part of the abomasum removed at the time of surgery.

INTRODUCTION

The adult ruminant depends almost completely on volatile, short-chain, fatty acids for energy; utilizes simple carbohydrates rather inefficiently; and has a lower blood glucose and a higher blood ketone concentration than the nonruminant. These characteristics are generally attributed to the development of rumen function and emphasize the complicating influence of the rumen microflora on detailed studies of nutritional requirements for ruminants.

During the course of investigations on carbohydrate digestion in young ruminants, it became desirable to conduct studies without interference from rumen fermentation. Rumenectomy techniques have been described in the literature [3, 4] but are limited in usefulness, since the omasum and some of the rumen-reticulum are left, thereby introducing the possibility of rumen regeneration [3] with subsequent microfloral development. The present report describes a procedure for the removal of rumen, reticulum, and omasum in 3-week-old calves.

Materials and Methods

The following procedure was used with Holstein-Friesian calves, 2 to 3 weeks old. The calves had been muzzled shortly after birth to prevent ingestion of roughage.

Feed was withheld 24 hours before surgery. Pentobarbital sodium was given slowly to effect, and the calf was placed in right lateral recumbency. A curved, 12-inch incision was made approximately 2 inches posterior to the last rib, and the peritoneal cavity was entered by routine procedure.[2] Hand retractors were used for proper exposure. The exposed viscera were kept thoroughly moistened with physiologic saline solution during the three-hour procedure. The spleen was freed of its dorsal, anterior, and ruminal attachments and removed after the splenic vessels were ligated. The celiac artery was then located and traced ventrally to the hepatic artery. The right and left ruminal, splenic, and omaso-abomasal arteries were ligated below the hepatic artery with No. 2 silk. The rumen was then freed of its attachments to the crura of the diaphragm, sublumbar muscles, and omentum, care being taken to avoid damaging the diaphragm. The rumen was then turned sufficiently to expose the portal system and permit ligation of the ruminal and omaso-abomasal veins.

Fig. 1—The rumen, reticulum, omasum, and part of abomasum (with omental attachments) removed from a calf at surgery.

Received for publication Feb. 17, 1961.

Contribution No. 40, Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ont.

The authors acknowledge the assistance of G. Hatina, R. Durnford, G. Sample, A. Ostapyk, and Dr. P. Lupien in performing the surgery. The preparation of the histologic sections by W. R. Settle is gratefully acknowledged.

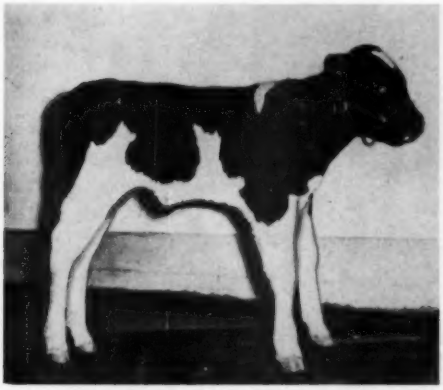


Fig. 2—Calf 1, one week after operation.

Next, the abomasum was carefully exteriorized. It was noticed that the distal third of the abomasum received adequate collateral blood supply from branches of the hepatic artery. Accordingly, intestinal clamps were placed across the abomasum at this point, the proximal segment was freed of omental attachments, bleeding vessels were ligated, and the proximal segment was excised. Next, intestinal clamps were placed approximately 2 inches from the cardia along the rumino-reticular wall. The second cut was made, the rumen and omasum were freed of remaining omental attachments, bleeding omental vessels were ligated, and the rumen, reticulum, omasum, and proximal portion of abomasum were removed (Fig. 1). Alignment of the cut ends was facilitated by using a special stainless steel intestinal clamp, without handles, on the abomasal end. The cut ends were united by a Connell suture line with No. 1 chromic catgut followed by a reinforcing row of continuous Lembert suture. The incision was closed by routine procedure.[2]

Animals were kept under 24-hour supervision for one week postoperatively. Electrolytes, dextrose, and protein hydrolysate were administered intravenously for maintenance, but no milk or water was permitted for 72 hours postoperatively. On the fourth day, approximately 50 ml. of milk and water was given at four-hour intervals; these amounts were gradually increased until the animals

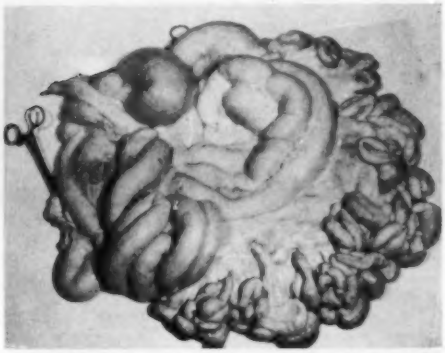


Fig. 4—Intestinal tract of calf 4 with stomach removed. Notice normal development and apparent lack of adhesions.

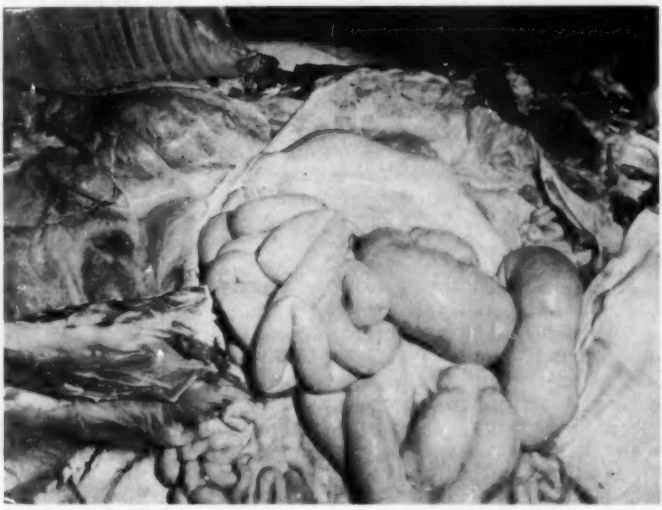


Fig. 3—Viscera of calf 4 at necropsy. Ribs and lung are reflected, exposing heart. Size of stomach (upper center) can be estimated by comparing with size of other organs.

Fig. 5—Stomach of calf 4 opened to show juncture of abomasum and rumen-reticulum.

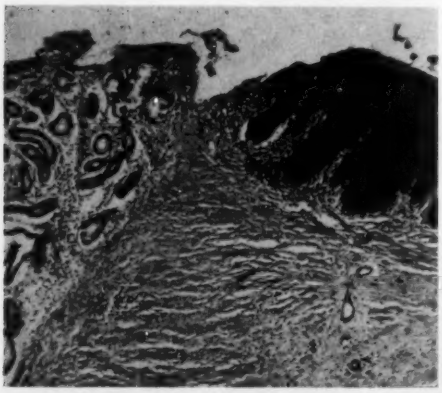


Fig. 6—Section through juncture of abomasum and rumen-reticulum of calf 4. Underlying connective tissue has firmly united the two tissues.

were on full feed, approximately three weeks after surgery. A four-hour feeding schedule was maintained throughout the life of each animal.

Results

It is difficult at this time to accurately estimate the mortality that may be expected with this surgical procedure. Four calves died during surgery while this technique was being developed; 2 of these died because the ligature on the celiac artery was placed incorrectly. Complete familiarity with the structural relationships of the celiac artery and its branches is essential, because it is often difficult to expose the area adequately during surgery and the ligature must be placed with complete accuracy. However, losses attributable to surgery may occur even if this procedure is used as described. One calf died ten days after surgery from rupture of the abomasal suture line after overfeeding, and 2 calves died four days postoperatively from

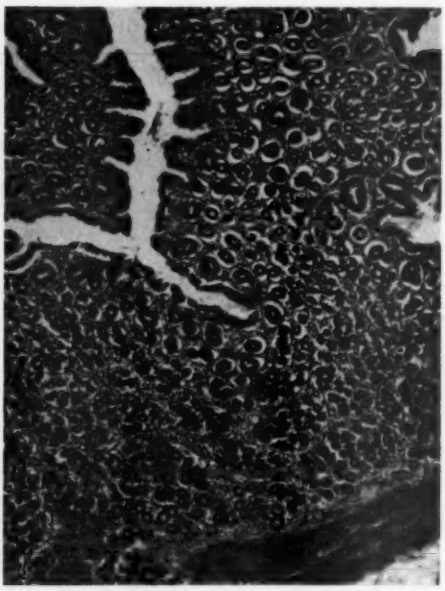


Fig. 7—Glandular development in abomasum of calf 4.

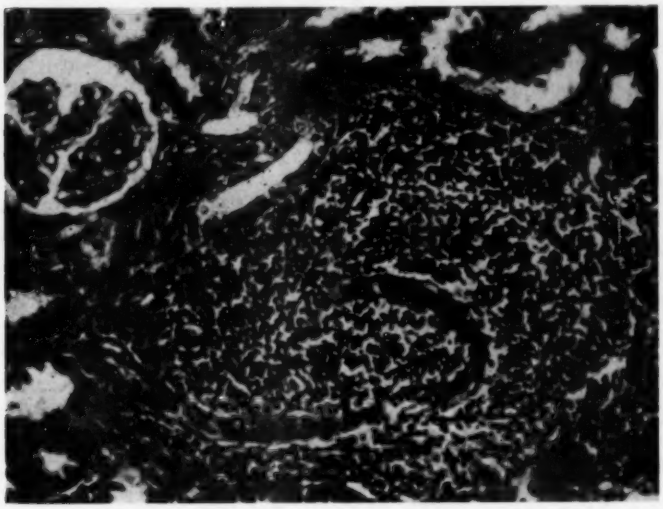


Fig. 8—Section through kidney cortex of calf 4. Notice area of lymphocytic cell infiltration.

perforation in the anterior duodenum that may have been the result of insufficient blood supply to that area.

The calves listed (Table 1) all had rapid postoperative recoveries and, in spite of their relatively small weight gains, were active and alert. Calf 1 is shown (Fig. 2) one week after surgery.

TABLE 1—Postoperative Survival and Weight Gains of Calves Following the Surgical Removal of the Forestomachs

Calf No.	Postoperative survival (days)	Postoperative weight gain (kg.)
1	99	18.0
2	102	10.5
3	112	9.0
4	199	39.5

No calf survived more than 200 days after surgery. Calf 1 died from a perforating ulcer of the stomach. Calves 2 and 3 died suddenly and the cause of death was not apparent at necropsy. Calf 4 died in convulsive seizure after having recovered spontaneously from two previous attacks. Calcium, magnesium, and dextrose had no effect on these seizures. Calf 4 is shown at necropsy (Fig. 3) with ribs and lungs reflected. The size of the stomach (top center) can be estimated by comparison with other organs. The intestinal tract (Fig. 4) had developed normally and there appeared to be no postsurgical adhesions. Figure 5 shows the simple stomach that was formed by joining abomasum to the oesophageal end of the rumen. The pyloric region, the remnant of the fundic gland region of the abomasum, and the area of attachment to the ruminal end are shown. The presence of fundic gland activity in the stomach was indicated by acid stomach secretions that readily coagulated milk. The ruminal part of the stomach had grown since surgery, emphasizing the difficulty in completely rumenectomizing calves unless a radical procedure is used.

Figure 6 shows a section taken through the juncture of abomasal and ruminal tissue. The two tissues were firmly united by underlying connective tissue. A section of the abomasal glands (Fig. 7) showed that glandular development was normal and that no injury resulted from deranging the abomasal blood supply. The liver (not shown) was normal both on gross and microscopic examination. In brain tissue there was slight meningeal congestion, but no other lesions were observed. The only abnormality was in the kidneys. The capsule was adherent and the surface was mottled. A few foci of lymphocytic cell infiltration were found in the cortex (Fig. 8). There was slight shrinkage of glomeruli, but the capillary lumens were patent. It is not known if these lesions were contributory to death of the animals.

Discussion

The results of this work indicate that calves may survive for a limited time after surgical removal of rumen, reticulum, omasum, and part of the abomasum. The cause of death in one calf was attributable to a perforating ulcer, but the cause of death in the other 3 calves was not apparent at necropsy. The possibility that nutritional deficiency contributed to the death of these animals cannot be ruled out; however, it seems remote, since they were fed a balanced diet supplemented with vitamins and minerals.[1] Further work is required to explain the failure of these calves to survive.

Since the surgery and postoperative care represent considerable expenditure of time, it is apparent that the usefulness of such calves is limited to specifically designed experiments. Furthermore, results obtained must be subject to careful interpretation in view of the fact that vagus innervation is destroyed and two-thirds of the abomasum removed at the time of surgery. Nevertheless, these calves were valuable in studying the effects of orally administered carbohydrate on blood glucose concentration.[1] The surgical removal of the forestomachs in calves may be useful in nutritional or metabolic studies that depend on the rigid exclusion of rumen microbial fermentation products.

References

[1] Lupien, P., Sauer, F., and Hatina, G. V.: Preliminary Studies with Simple Stomached Calves. J. Dai. Sci., (1961): In press.

[2] Markowitz, J.: Experimental Surgery. 2nd ed. Williams & Wilkins Co., Baltimore, (1949): 70–107.

[3] Trautman, A., and Schmitt, J.: Beiträge zur Physiologie des Wiederkäuer Magens. II. Mitteilung. Regeneration von Pansen und Haube nach Exstirpation dieser Organe. Arch. Tierernährung und Tierzucht, 7, (1931): 421–435.

[4] Wise, G. H.: The Physiological Role of the Rumen of the Young Bovine as Indicated by the Growth and Blood Composition of a Rumenectomized Calf. J. Dai. Sci., 29, (1946): 543–544.

SUMMARIO IN INTERLINGUA

Un Technica pro le Ablation Chirurgic del Antestomachos de Vitellos

Quatro vitellos de 3 septimanas de etate superviveva, pro plus que tres menses, un operation chirurgic que consisteva in le ablation del rumine, del reticulo, del omaso, e de un parte del abomaso.

Al necropsia, un perforante ulcere gastric esseva trovate in 1 del vitellos. Le causa de morte in le altere 3 non pareva esser directemente relationate con le intervention chirurgic.

Le utilitate de iste vitellos pro objectivos experimental esseva restringite per le facto que illos non superviveva le operation per plus que sex menses e, in plus, per le facto que al operation le innervation gastro-vagal esseva destruite e un parte del abomaso esseva removite.

Serologic Studies Suggestive of *Leptospira hardjo* in a Beef Herd

M. A. Ramirez, D.V.M., M.S.; R. C. Allen, Ph.D.:
W. R. Van Dresser, D.V.M.; D. F. Watson, V.M.D.

SUMMARY

When leptospirosis occurred in a herd of 44 steers, blood samples were obtained once a month and all steers were killed three months after signs had first been noticed. Leptospires were not isolated from the steers but hamsters inoculated with urine from the steers developed a significant titer to the *hebdomadis* serogroup. The serologic variations obtained proved that the agglutination-lysis tests alone do not provide exact information concerning the infecting serotype, that for this it is necessary to carry cross-absorption studies. The results of the cross-absorption studies seemed to indicate that *Leptospira hardjo* might be the serotype responsible, and at the same time serums with titers to *Leptospira sejroe* were found. The results also suggested that the steers were infected with both *Leptospira hardjo* and *Leptospira pomona*.

INTRODUCTION

In a large percentage of the bovine serum samples submitted to this laboratory for serologic testing, predominant titers to the *hebdomadis* serogroup have been demonstrated. When these serums were tested against 3 serotypes of the *hebdomadis* serogroup, *Leptospira hardjo* (Hardjoprajitno),* *Leptospira sejroe* (Mallersdorf)** and *Leptospira hebdomadis*, cross-reactions to two or more of the above serotypes were observed. The purpose of this work is to report clinical and serologic studies on a beef herd in which agglutinins to the *hebdomadis* serogroup and to *L. pomona* were found.

Received for publication Nov. 21, 1960.

From the Department of Veterinary Science, Virginia Polytechnic Institute, Blacksburg, Va.

* The strain of *Leptospira hardjo* (Hardjoprajitno) was provided by Dr. E. E. Roth, Department of Veterinary Science, Louisiana State University, Baton Rouge.

** Obtained from Veterinary Division, Walter Reed Army Institute of Medical Research, Washington, D.C.

Leptospira pomona is now recognized to be the most important cause of leptospirosis in the bovine and porcine species.[13] The public health significance, economic importance to the livestock industry, and pathogenicity of this organism have attracted the attention of research men to the extent that more is known about this serotype than any of the other leptospires.[10] Information concerning the *hebdomadis* serogroup and its relationship to the problem of bovine leptospirosis is limited, however.

For many years, investigators have been concerned with the presence in the bovine serums of agglutinins detected with antigens representing members of the *hebdomadis* serogroup. Galton *et al.*[5] first reported the presence of this agglutinin in bovine serums in the United States in 1952. The agglutinins present were active against *L. sejroe*. Similar reports from various areas of the country have since appeared.[2, 3, 6, 7, 9]

During the past 8 years, some experimental work and several surveys have been conducted in an attempt to secure more information about the problem.[2, 3, 5, 7, 16] The presence of agglutinins against *L. sejroe* indicated that the animals had been exposed to this serotype. The etiologic agent, however, was not isolated. Roth [11] isolated *L. hardjo*, a serotype antigenically related to *L. sejroe*, from Louisiana cattle in which agglutinins against *L. sejroe* had been demonstrated. This was the first successful isolation of *L. hardjo* from cattle. The growth of the organism upon primary isolation apparently required modification of the Stuart's medium by the addition of 0.3% agar (Difco).

At Virginia Polytechnic Institute, several of 44 steers 12 to 16 months old, on a nutritional experiment, became ill and had elevated body temperatures that persisted 2 to 4 days. They had diarrhea, inflammation of the superficial lymph nodes, and loss of condition.

Materials and Methods

One steer was killed and examined grossly for lesions. Blood samples were drawn from the re-

maining 43 steers at monthly intervals for serologic examination. All of the steers were killed and examined 3 months after the first clinical signs were noticed. One milliliter of urine from each steer was injected intraperitoneally into each of 2 hamsters. After the third postinoculation day, blood samples were taken daily from the hamsters' hearts. One drop of blood was examined under dark-field illumination and 2 drops were placed into each of two tubes containing Fletcher's medium.

Leptospiral strains for the agglutination-lysis test were grown in Bacto-Stuart's Medium Base[14] (Difco) containing 10% rabbit serum. Cultures of leptospires for antigen were incubated at 30 C. All antigens used contained approximately 1.8×10^8 leptospires as determined in a Petroff Hauser counting chamber.[15]

Serologic titers were determined by the agglutination-lysis test and expressed as the dilution of the last tube showing agglutination of 50% of the cells. Serial dilutions of serums were made with sterile buffered 0.85% salt solution (pH 7); 0.2 ml. of each serum dilution was mixed with 0.2 ml. of viable *Leptospira* antigen to give a final dilution of 1:100, 1:400, 1:1,600, 1:6,400, 1:25,600, and 1:102,400. The dilutions were shaken for one minute and incubated for two hours at 37 C. The agglutination test was read using a darkfield microscope at a magnification of x 150.

Absorbing antigens were prepared from 10-day-old cultures grown in the basal medium. The cultures were treated with formalin to a final concentration of 0.25% and centrifuged at 4,000 x g at 0 C. for one hour. The supernatant fluid was discarded and the packed cells resuspended with formolized buffered 0.85% salt solution (pH 7.4) to a density approximating the fourth tube in the McFarland series. Four parts of the absorbing antigens were added to one part of the test serum.

The mixed samples were placed in a Dubnoff shaker for two hours at 37 C. and then held at 4 C. for 12 hours in a refrigerator. All serums were absorbed 3 times by this procedure. Microscopic agglutination test with the absorbed serums was performed by the procedure described previously, except that the initial dilution was 1:40.

Results

Body temperatures up to 106 F. persisted for several days. The clinical signs observed were enlargement of the superficial lymph nodes, diarrhea, loss of appetite, roughness of the hair, and loss of condition. There were no deaths but the morbidity was high. Four weeks after initial signs of disease were noticed, necropsy of one steer revealed white spots, varying in size from 1 to 4 mm. in diameter, spread over the cortex of the kidneys. These spots were irregular in shape and many extended into the medulla. Histologically, there were large masses of

TABLE 1—Agglutinin Absorption Studies on Eight Serums Representative of 43 Steers, Using Various Leptospiral Strains

Titers before absorption				Titers following absorption with *L. sejroe*				Titers following absorption with *L. hardjo*				Titers following absorption with *L. pomona*			
L.p.*	L.h.	L.heb.	L.s.	L.p.	L.h.	L.heb.	L.s.	L.p.	L.h.	L.heb.	L.s.	L.p.	L.h.	L.heb.	L.s.
Neg.	2,560	640	2,560	Neg.	40	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	2,560	640	2,560
2,560**	2,560	1,280	2,560	2,560	80	Neg.	Neg.	2,560	Neg.	Neg.	Neg.	Neg.	2,560	1,280	2,560
Neg.	1,280	320	1,280	Neg.	80	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1,280	320	1,280
1,280	2,560	640	1,280	1,280	160	Neg.	Neg.	1,280	Neg.	Neg.	Neg.	Neg.	2,560	640	1,280
Neg.	1,280	640	1,280	Neg.	40	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1,280	640	1,280
2,560	1,280	320	2,560	2,560	80	Neg.	Neg.	2,560	Neg.	Neg.	Neg.	Neg.	1,280	320	2,560
Neg.	1,280	1,280	1,280	Neg.	80	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	1,280	1,280	1,280
Neg.	5,120	1,280	5,120	Neg.	320	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	5,120	1,280	5,120

* L.p = *L. pomona*, L.h. = *L. hardjo*, L.heb. = *L. hebdomadis*, L.s. = *L. sejroe*; ** reciprocal of the titer.

lymphoid cells infiltrating the cortex and medulla; there was also thickening of Bowman's capsule and discrete fibrosis. Some of the tubules appeared distended and filled with protein-like material.

The cells of the tubules had undergone various degrees of degeneration. Before being killed, this particular animal had a positive titer of 1:400 to *L. pomona* and 1:1,600 to *L. sejroe*.

Figure 1 shows the total number of steers with positive agglutination-lysis reactions to the *Leptospira* antigens over a period of three months. Of 43 steers, 38 had positive titers to *L. hardjo*; of these 38 (*L. hardjo*-positive), 35 also had titers to *L. sejroe* and 6 had titers to *L. pomona*. Three of 43 steers had titers to *L. pomona* alone. Serums from naturally infected cattle had titers ranging from 1:100 to 1:6,400 to both the *hebdomadis* and *autumnalis* serogroups. *Leptospira hardjo, L. sejroe,* representing the *hebdomadis* serogroup, and *L. pomona,* representing the *autumnalis* serogroup, were the antigens used. Over this period of time the animals that became ill developed higher titers to *L. hardjo* and to *L. sejroe*. This correlation was not consistently found with *L. pomona*. *Leptospira canicola, L. autumnalis,* and *L. icterohaemorrhagiae* did not produce agglutination titers above 1:100.

The average serologic titer of the 43 steers over a 3-month period against *L. pomona, L. sejroe,* and *L. hardjo* is shown (Fig. 2). Titers to *L. pomona* remained low, whereas titers to *L. sejroe* went from 1:400 in May to 1:3,000 in June and to 1:200 for July. Titers to *L. hardjo* were in the order of 1:200 for the first two months but during the last month they went up to 1:2,900. The individual serologic pattern over the three-month period varied considerably. This is important because it demonstrates that the serologic test alone will not always provide evidence as to the infecting serotype. Results of the agglutinin absorption studies on eight bovine serum samples having *hebdomadis–autumnalis* serogroup cross reactions are shown (Table 1). When serums were absorbed with *L. sejroe,* agglutinins to *L. hebdomadis* and *L. sejroe* were removed, while agglutinins to *L. hardjo* persisted in low titers. Absorption with *L. hardjo* removed the agglutinins to the three serotypes used.

Leptospires were not isolated from the hamsters inoculated with urine from the

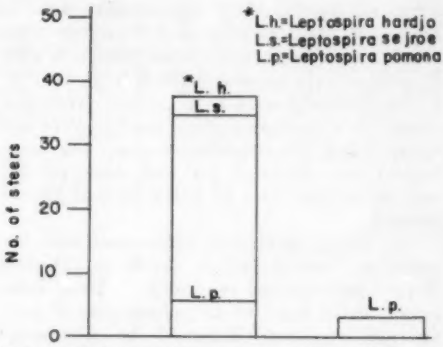


Fig. 1—Total number of steers with positive agglutination-lysis reactions to Leptospira antigens over a three-month period.

steers, nor were they seen under dark-field microscope or isolated in Fletcher's medium, but serologic examination of hamster serums 20 days after inoculation revealed the presence of titers to the *hebdomadis* serogroup.

Discussion

These results seem to indicate that the serotype involved might have been *L. hardjo,* since the agglutinins for *L. hardjo* are not completely removed when absorbed with *L. sejroe*. Following absorptions

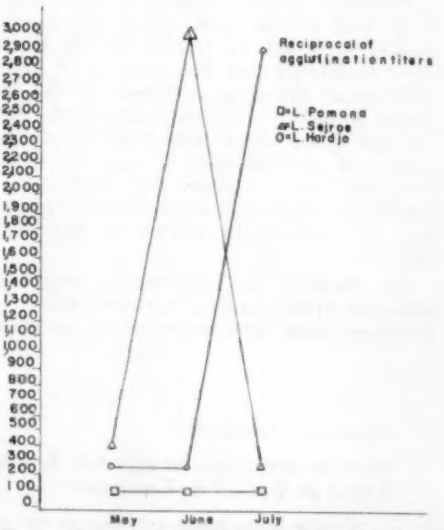


Fig. 2—Average serologic titer of the 43 steers over a three-month period.

with *L. hardjo*, the agglutinins for *L. hardjo*, *L. hebdomadis*, and *L. sejroe* were removed. When serums were absorbed with *L. pomona*, the serologic titers to *L. hardjo*, *L. hebdomadis*, and to *L. sejroe* were not changed. Cross absorption studies were not made using *L. autumnalis* since the serologic titers obtained for this serotype did not exceed the titer of 1:100 in 3 of the 44 serums.

The minor antigenic differences seen between *L. hardjo* and *L. sejroe* might produce "paradoxical reactions." This, however, would have to be substantiated with further research designed to determine whether the nonspecific co-agglutinins would eventually disappear or diminish considerably. Wolf [16] has shown that *L. sejroe* and presumably *L. hardjo* are not antigenically related to *L. pomona*.

The steers in this study had not been vaccinated against leptospirosis. The possibility exists that they might have been infected with both *L. hardjo* and *L. pomona*. Infection with two serotypes of *Leptospira* has been experimentally produced.

We were unable to isolate leptospires in Fletcher's medium or to see them under dark-field illumination from hamster urine or blood. After this work was completed, Roth [11] reported isolation of *L. hardjo* in Stuart's medium modified by the addition of 0.3% agar (Difco).

Failure to recover leptospires from hamsters and guinea pigs following experimental infection has raised doubts as to their suitability for this purpose.[7,8] The mildness of the signs developed by the infected hamsters may have indicated that the leptospiral strain used possessed a low degree of pathogenicity or that the hamsters were more resistant than cattle. The possibility exists that more virulent strains may be present in naturally infected animals.

The titers to the *hebdomadis* serogroup obtained from hamster serums following intraperitoneal inoculation of urine from steers indicates that the steers were infected at the time of slaughter. A further indication of this was the fact that gross and microscopic lesions suggestive of leptospirosis were found in the kidneys of some steers.

References

[1] Alexander, Aaron D.: The Laboratory Diagnosis of Leptospirosis. Division of Veterinary Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington 12, D.C., 1959.

[2] Byrne, R. J., and Chambers, F. C.: A Serological Survey of Leptospiral Antibodies in Maryland Cattle. J.A.V.M.A., 134, (June 1, 1959): 498–502.

[3] Ferris, D. H., Hanson, L. E., Alberts, J. O., Calhoun, J. C., and Marlow, R.: Epizootiological Studies on *Leptospira* in Illinois. I. Serological Tests in Illinois Deer. 1957. Proc. CDC Conference for Teachers of Veterinary Public Health and Preventive Medicine and Public Health Works (June 17–18, 1958): 354–361.

[4] Führer, F.: Über die Bedeutung der Mitreaktionen artverschiedener Leptospirenantigene bei der Auswertung serologischer Leptospiroseergebnisse für Klinik und Praktis. Ztsch. Immunitätsforsch., 108, (1950a): 278.

[5] Galton, M. M., Acree, J. A., Lewis, A., and Prather, E. C.: Leptospirosis in Domestic Animals in Florida with Reference to Cattle. J.A.V.M.A., 128, (Jan. 15, 1956): 87–91.

[6] Hale, Maurice W.: Laboratory Report on Leptospirosis in Georgia, 1957. J.A.V.M.A., 133, (Aug. 15, 1958): 196–197.

[7] Hoag, W. G.: The Presence of *Leptospira sejroe* Agglutinins in Cattle Serum. Am. J. Vet. Res., 18, (Jan., 1957): 50–52.

[8] Hoag, W. G.: Unpublished data, Virginia Polytechnic Institute, Blacksburg, Va., 1952.

[9] Lang, R. W., and Morse, E. V.: Serological Cross Reactions Among Leptospiras Observed with Sera from Animals Infected with *L. pomona*. J. Immunol., 82, (1959): 471.

[10] Mengies, R. W., Galton, M. M., and Habermann, R. T.: Cultural and Serologic Studies on Four Dogs Inoculated with Two Leptospiral Serotypes, *Leptospira pomona* and *Leptospira canicola*. Am. J. Vet. Res., 21, (May, 1960): 371–376.

[11] Morse, E. V.: New Concepts of Leptospirosis in Animals. J.A.V.M.A., 136, (March 15, 1960): 241–246.

[12] Roth, E. E., and Galton, M.: Isolation and Identification of *Leptospira hardjo from* Cattle in Louisiana. Am. J. Vet. Res., 21, (May, 1960): 422–427.

[13] Simmons, J. S., and Gentzkow, C. J.: Method of Determining Bacterial Content by Direct Count. Laboratory Methods of U.S. Army, (1944): 404.

[14] Steele, J. H.: Epidemiology of Leptospirosis in the United States and Canada. J.A.V.M.A., 136, (March 15, 1960): 247–252.

[15] Stuart, R. D.: The Preparation and Use of a Single Culture Medium for Leptospirae. J. Path. & Bact., 58, (1946): 343–349.

[16] Wolf, J. W.: The Classification of Pathogenic Leptospires. Symposium on the Leptospirosis. U.S. Government Printing Office, Washington, D.C., 1953.

[17] Yager, R. H.: Leptospirosis in the United States Today. Symposium on the Leptospirosis. Med. Sci. Publ. No. 1, Army Med. Serv. Grad. School, (1952): 222–223.

SUMMARIO IN INTERLINGUA

Studios Serologic, Suggerente le Presentia de *Leptospira hardjo* in Grege de Bestial de Consumo

Quando leptospirosis occurreva in un grege de 44 boves, specimens de sanguine esseva obtenite a intervallos de un mense, e omne le animales esseva sacrificate tres menses post que

le prime signos del condition habeva essite notate. Leptospiras non esseva isolate ab le boves, sed hamsters inoculate con urina de illos disveloppava titros significative anti le sero-gruppo de *Leptospira hebdomadis*. Le variationes serologic obtenite provava que tests de agglutination e lyse per se non provide precise informationes in re le serotypo que causa le infection e que, pro obtener tal informationes, il es necessari interprender studios de absorption cruciate. In le presente caso, le resultatos de tal studios de absorption cruciate pareva indicar que *L. hardjo* esseva possibilemente le serotypo responsabile, sed al mesme tempore seros con titros anti *L. sejroe* esseva trovate. In plus, le resultatos suggereva que le boves esseva inficite tanto con *L. hardjo* como etiam con *L. pomona*.

Ruminant Urolithiasis. IV. Ultrafiltration, Electrophoretic, and Chromatographic Studies on Urinary Biocolloids of Sheep

C. E. Cornelius, D.V.M., Ph.D.; J. A. Bishop, B.S.; L. R. Berger, Ph.D.; J. Pangborn, M.A.

SUMMARY

The total nondialyzable urinary biocolloids of sheep were isolated by ultrafiltration procedures and separated into two fractions as either soluble or insoluble in 1M NaCl. Upon changing wethers from alfalfa pellets to concentrate pellets, the concentration of the urinary salt-soluble biocolloids increased with a decrease in urine volume. No significant variations occurred in the salt-soluble biocolloid excreted per 24 hours per kilogram of body weight or in the filterable salt-insoluble "uromucoid" fraction. Significant increases occurred in the concentration and daily output of the salt-soluble urinary colloids per kilogram of body weight of sheep following subcutaneous implantation of diethylstilbestrol. Urinary biocolloids and serum proteins were fractionated using both electrophoresis and diethylaminoethyl-cellulose anion-exchange resins.

INTRODUCTION

Information concerning the total nondialyzable solids (TNDS) in ruminant urine is nonexistent. Previous studies on urinary colloids in sheep have been confined to a protein-bound hexosamine fraction[14] and a benzoate-adsorbed heterogeneous glycoprotein fraction isolated in this laboratory.[5] An increased concentration of this urinary glycoprotein fraction and the blood seromucoid was observed in sheep given a "calculi-provoking" diet. During experimental ovine calculosis, significant increases in neutral glycoprotein were observed by histochemical techniques throughout the urinary system as well as in the matrix of phosphatic calculous material.[4] The intimate interrelationship between matrix and mineral in uroliths from various domestic species has recently been reported.[3]

Ultrafiltration of urine and subsequent electrophoretic identification of urine colloids, as studied by Boyce and coworkers in man,[1,2] has revealed a salt-insoluble (1M NaCl) glycoprotein, "uromucoid" (T and H glycoprotein[13]), and salt-soluble proteins with properties of certain serum proteins. They observed that the TNDS was three to 13 times greater in the urine of patients with calculous disease than in normal urine.

The purpose of this investigation was threefold: (1) to examine quantitatively and qualitatively the TNDS in the urine of sheep both on differing diets and following subcutaneous implantation of diethylstilbestrol; (2) to compare the salt-soluble (1M NaCl) urinary biocolloids with serum proteins electrophoretically and on diethylaminoethyl-cellulose anion-exchange resins; (3) to purify and determine the homogeneity of salt-insoluble ovine *"uromucoid"* (T and H glycoprotein).

Materials and Methods

The basic control diet was alfalfa pellets containing 1.43% K, 0.24% P, 1.28% Ca, and 0.18% Mg. To measure the effect of concentrate pellets on urinary colloidal excretion, a pelleted diet of 15% alfalfa meal, 35% beet pulp, 20% linseed meal, 15% oats, and 15% wheat was fed. Analysis of the concentrate ration revealed a mineral composition of 0.88% K, 0.34% P, 0.70% Ca, and 0.36% Mg. The effect of stilbestrol implantation on urinary colloids excreted was studied, using either 15 or 60 mg. of diethylstilbestrol by means of pellets implanted subcutaneously at the base of the ear.

The 24-hour urine samples were collected in ice baths from sheep in metabolism stalls as previously described.[5] Thymol crystals were added to prevent bacterial growth. Each urine aliquot was

Received for publication Jan. 24, 1961.

From the Department of Clinical Pathology, School of Veterinary Medicine, University of California, Davis. Dr. Berger is now with the Department of Microbiology, University of Hawaii, Honolulu.

This study was supported in part by a grant (USPH-A-3296) from the National Institutes of Arthritis and Metabolic Diseases, and Regional Research Funds from the United States Department of Agriculture for Cooperative Research on Western Regional Project 41.

acidified to pH 6.8 with 4 N acetic acid under constant stirring to force any crystalline phosphates into solution and subsequently was filtered through a coarse filter paper * to remove cells originating in the urinary system. Some of the "uromucoid" was also retained on the filter paper in this step. The filtered urine was next placed in cellophane dialyzing tubing ** and dialyzed against repeated changes of distilled water until the specific conductance was less than 20 micromhos per centimeter. The dialyzed specimens were subjected to ultrafiltration procedures as described previously by Gorbman [6] in a stainless steel filtration apparatus† under 80 p.s.i. of nitrogen, using collodion membranes‡ with a pore size of 10 to 20 mμ. Following ultrafiltration, the colloidal gel was washed off the membrane with 10 ml. of 1M NaCl and transferred to a 15 ml. conical centrifuge tube. Following centrifugation at 1,500 g for 10 minutes, the small amount of insoluble residue was washed with saline solution and again centrifuged. The saline washings were transferred to the soluble fraction. Both soluble and insoluble fractions were dialyzed in ⅝ inch diameter dialyzing tubing against distilled water until free from ions, then lyophilized and weighed.

The lyophilized soluble and insoluble residues were analyzed for the following: protein content by the biuret reaction [16] or Folin-Ciocalteu reagents [7]; sialic acid by the diphenylamine method of Winzler,[17] using a standardized solution of acetyl-sialic acid; protein-bound hexose by the orcinol reaction [17]; and hexosamine by Schloss' modification of the method of Elson and Morgan.[10]

Electrophoresis of urinary colloids was performed by conventional techniques in an electrophoresis apparatus,# using a 2-ml. microanalytical cell. The diagonal slit and cylindrical lens system as recommended by Philpot [9] was used with special contrast§ film. Tracings of negatives were made because of a lack of uniform exposure in the original photographs from interference of urinary pigments. Lyophilized salt-soluble urinary colloids and serum proteins were dissolved and dialyzed against 0.1M veronal buffer at pH 8.6 prior to electrophoresis in this buffer system. Soluble urinary colloids were diluted to a concentration of 2% protein prior to electrophoresis due to the high concentration of interfering bound or adsorbed pigments. Serum proteins were analyzed electrophoretically at a concentration of 4%. All protein fractions in veronal buffer were separated using 240 to 255 volts at 4.5 to 5.0 ma. The lyophilized insoluble urinary colloid was dialyzed for 72 hours against 0.1M glycine buffer at pH 12.0 as described previously by Boyce *et al.*,[2] and the resulting soluble supernatant fraction following centrifugation was analyzed electrophoretically (160v, 9 ma) in this buffer system for 130 minutes at a concentration of 0.8 to 1.0 Gm% of protein. Serum proteins were treated identically at pH 12.0 and analyzed electrophoretically for comparison.

Serum proteins and salt-soluble urinary colloids were analyzed and compared chromatographically at 4 C. on an anion-exchanger, diethylaminoethyl cellulose (DEAE-C)* by techniques similar to those described by Sober *et al.*[11] Serum samples were first dialyzed against distilled water, lyophilized, and resuspended in a smaller volume of distilled water before application to the column. Salt-soluble urinary colloids from several sheep were pooled before being suspended in a few milliliters of water and applied to the DEAE-C column. The salt-insoluble pooled fractions were rendered "soluble" by suspension in 0.1M glycine buffer at pH 12.0 as for electrophoretic analysis. When further concentration was needed, colloid was lyophilized and resuspended in distilled water before application to the columns. All protein fractions were slowly added to the surfaces of the DEAE-C columns and forced into the solid phase with a small amount of N_2 pressure. One to 5 ml. of solution I (Fig. 3) were next added, and then a small amount of DEAE-C was used as a covering layer prior to final column assembly. Gradients were made in the usual way from a reservoir to a mixing chamber. Gradient solutions were designated in this report by their theoretical limit concentrations although never completely reached. Elution gradients, column size, the amount of protein applied and adsorbent used for the separation of ovine serum proteins and salt-soluble urinary colloids were similar to those reported by Sober *et al.*[11] All gradients are reported with the effluent diagrams in the section on results. Effluent fractions were routinely examined in a spectrophotometer ** and expressed as milligrams of protein per milliliter by multiplying 1.5 times the optical density at 280 mμ. Standardization for protein was performed using bovine albumin with the biuret [16] and Folin-Ciocolteu procedures [7]

Effluent fractions which were dialyzed free of conductivity, lyophilized, and reconstituted to approximately 5% protein were examined by paper electrophoresis. Electrophoretic analysis was performed on a Spinco Model R electrophoresis cell using either 0.1 or 0.05M sodium diethylbarbital or 0.05M sodium tetraborate buffer systems at pH 8.6. Strips were stained with bromphenol blue and migration distances were compared to those of ovine serum proteins.

Results

The dry weights of the lyophilized 1M NaCl-soluble and -insoluble biocolloids re-

* No. 617, Eaton-Dikeman, Mt. Holly Springs, Pa.

** No. 4465-A2, Arthur H. Thomas Co., Philadelphia, Pa.

† Model 4000, Hormann and Co., Newark, N.J.

‡ No. LSG-60, Schleicher and Schuell Co., Keene, N.H.

Klett electrophoresis apparatus, New York, N.Y.

§ Contrast process panchromatic film, Eastman Kodak Co., Rochester, N.Y.

* Biorad Laboratories, Richmond, Calif.

** Beckman DU Spectrophotometer, South Pasadena, Calif.

covered following ultrafiltration are presented (Table 2). In trial I, urine was first collected from a group of 15 sheep consuming alfalfa pellets. Subsequently, during the next month, urine was collected when they were fed a concentrate pelleted ration. During this second period, a significant increase was observed in the concentration (mg./liter) of salt-soluble urinary biocolloids ($P = 0.02$), and in the blood seromucoid concentration ($P = 0.01$). No statistically significant change was observed in the salt-soluble urinary colloid when expressed as milligrams per day per kilogram of body weight or in the filterable salt-insoluble "uromucoid" fraction. Much of the salt-insoluble fraction was lost due to the filtration of the urine through filter paper. The 24-hour urine volumes decreased when the sheep were given the concentrate pellets ($P = 0.05$). Atmospheric temperatures were not greatly elevated at the times of urine collection in either period with mean minimum and maximum winter temperatures for the alfalfa and concentrate feeding periods at 3 to 21 C. and 6 to 13 C., respectively. The significant reduction in urine volume per kilogram of body weight during concentrate feeding was most likely the result of the change in diet as previously reported by Newsom [8] and not a result of changes in environmental temperature. The lower urine volumes in turn are obviously related to the higher concentration of salt-soluble colloid in the urine. The concentration of soluble colloid in 12 of 13 wethers in which collections were made increased on the average by a factor of 1.91 when they were fed concentrate pellets.

In trials II and III, urinary colloids were collected from 18 wethers: 6 controls, 6 given 15 mg. of diethylstilbestrol implants subcutaneously, and 6 given a 60-mg. implant (see Table 1). Trial II consisted of urine collections one to nine weeks after implantation while trial III collections were made on the same wethers ten to 15 weeks after implantation. Remarkable increases above the levels in the control group were observed in the salt-soluble urinary colloid in about one-half of the wethers given diethylstilbestrol at either the 15- or 60-mg. level. The number of animals was too low in trials II and III for any true statistical representation of an ovine population. However, no levels of salt-soluble urinary colloids in any control group or in the previous trials with concentrate feeding approached the elevations encountered in these wethers following hormone implantation. Mean daily minimum and maximum environmental temperatures for trial II were 7 to 24 C., while in trial III temperatures were 14 to 32 C. Salt insoluble colloid excretion did not vary significantly. Blood seromucoid levels in trials II and III were difficult to interpret due to the limited number of sheep in each group. The 6 wethers given 15-mg. implants of diethylstilbestrol in trial II, however, had lower seromucoid levels than their controls ($P = 0.01$).

Partial organic analyses of ovine urinary colloids are presented (Table 2). These analyses suggest that the heterogeneous salt-soluble fraction contained more protein (as calculated from its tyrosine equivalents [7]) and sialic acid than the salt-insoluble "uromucoid" fraction. Considerable protein-bound carbohydrate was present in both fractions.

Phosphotungstic acid (PTA) has been used by some investigators [14] to precipitate a "protein-bound hexosamine" fraction from perchloric acid filtrates of ovine urine. Urine was examined before and after dialyses against distilled water and following ultrafiltration through collodion membranes of a pore size 10 to 20 mμ to better characterize this heterogeneous fraction. The results are shown (Table 3). It is quite apparent if the "tyrosine"-reacting substances from untreated urine were assumed to be only of protein origin as presented in Table 3, column 2, overestimates of the urinary protein concentration would be made. Since approximately one-half of the hexosamine and the majority of the "tyrosine-reactive" substances are dialyzable and not firmly bound, it is questionable what percentage of this PTA fraction would truly represent conjugated urinary glycoproteins.

Electrophoretic analyses of four samples of salt-soluble urinary colloids prepared by ultrafiltration of urine pooled from various sheep revealed the presence of similar proteins to those previously reported by Boyce *et al.*[1, 2] and Webb *et al.*[16] in normal human urine. Tracings of electrophoretic patterns obtained in 0.1M veronal buffer at pH 8.6 are compared to the normal serum proteins of sheep (Fig. 1). In all electrophoretic analyses of salt-soluble urinary colloid from sheep fed either the concen-

TABLE 1—Urinary Biocolloid Excretion in Wethers on Differing Diets and Following Diethylstilbestrol Implantation

Trial No.	No. of sheep	Diet	Treatment	Age (months) at time of collection $\bar{X}$*	Weight (kg.) $\bar{X}$	1M NaCl-soluble biocolloid		1M NaCl-insoluble biocolloid**		Volume of urine	Blood seromucoid
						mg./liter	mg./day/kg.†	mg./liter	mg./day/kg.	(ml./day/kg.)	(mg./100 ml. serum)
						$\bar{X}$ δ	$\bar{X}$ δ	$\bar{X}$ δ	$\bar{X}$ δ	$\bar{X}$ δ	$\bar{X}$ δ
I	15	Alfalfa pellets	....	5.9 (5.0–7.7)	30	56.7 ± 27.4 (22–118)	2.16 ± 0.26 (1.12–3.65)	7.4 ± 2.9 (3.0–12.0)	0.272 ± 0.118 (0.107–0.464)	40.6 ± 9.7 (22.7–53.5)	28.7 ± 9.9 (13–45)
I	14	Concentrate pellets	....	7.3 (6.0–9.0)	35	85.3 ± 43.6 (26–151)	2.54 ± 0.37 (1.4–5.7)	9.2 ± 5.0 (1–17)	0.309 ± 0.137 (0.115–0.662)	33.0 ± 11.0 (17.5–56.0)	39.2 ± 11.1 (12–57)
II	6	Alfalfa pellets	Controls	10.7 (9.7–11.5)	44	77 ± 6.7 (53–104)	2.77 ± 0.64 (2.16–3.56)	6.6 ± 3.4 (2–12)	0.323 ± 0.114 (0.138–0.440)	36.7 ± 8.7 (26.4–45.0)	36.5 ± 9.6 (20–49)
II	6	Alfalfa pellets	9–62 days after 15 mg. estrogen	10.7 (9.7–11.5)	39	174 ± 56 (71–425)	5.52 ± 4.80 (1.51–14.8)	8.3 ± 4.8 (4–15)	0.238 ± 0.164 (0.072–0.524)	47.6 ± 10.8 (34.9–64.1)	24.5 ± 9.0 (15–38)
II	6	Alfalfa pellets	12–67 days after 60 mg. estrogen	10.7 (9.7–11.5)	43	116 ± 25 (55–209)	5.24 ± 3.34 (2.94–11.8)	4.3 ± 2.6 (1–9)	0.305 ± 0.160 (0.073–0.553)	50.2 ± 23.0 (20.6–88.0)	34.3 ± 13.1 (21–52)
III	6	Alfalfa pellets	Controls	12.5 (12.0–13.2)	46	75 ± 31 (40–129)	3.71 ± 1.19 (1.85–5.50)	8.0 ± 4.4 (3–15)	0.323 ± 0.184 (0.175–0.658)	47.1 ± 16.2 (32.1–72.6)	32.0 ± 10.6 (23–52)
III	6	Alfalfa pellets	75–105 days after 15 mg. estrogen	12.5 (12.0–13.2)	47	228 ± 231 (56–673)	5.59 ± 3.25 (1.64–9.98)	12.6 ± 15.1 (1–39)	0.235 ± 0.106 (0.52–0.427)	33.3 ± 18.1 (11.1–63.8)	34.4 ± 16.1 (6–45)
III	5	Alfalfa pellets	75–110 days after 60 mg. estrogen	12.5 (12.0–13.2)	50	182 ± 132 (25–377)	6.03 ± 4.86 (1.36–10.80)	6.4 ± 3.2 (1–9)	0.213 ± 0.115 (0.050–0.354)	37.1 ± 10.4 (31.4–54.3)	26.0 ± 8.9 (18–41)

* $\bar{X}$ = mean; δ = standard deviation. The range is also reported.
** This fraction represents that which was recovered following filtration through paper (*see* Materials and Methods).
† Body weight.

TABLE 2—Partial Organic Analyses of Ovine Urinary Colloids—Per Cent of Dry Weight

Constituent	No. of Samples	Diet	% protein (biuret) $\bar{x}$	% protein (biuret) R	% tyrosine [7] $\bar{x}$	% tyrosine [7] R	% sialic acid $\bar{x}$	% sialic acid R	% hexose $\bar{x}$	% hexose R	% hexosamine x	% hexosamine R
		Pellet										
Total non-dialyzable solids	4	Alfalfa	80.0	(68.1–89.4)	6.9	(5.7–8.8)	5.4	(4.1–6.4)	6.8	(5.7–8.1)	5.8	(4.5–7.2)
1M NaCl soluble colloids	2	Alfalfa	80.6, 90.6		8.8, 7.1		5.1, 5.4		7.8, 6.0		6.5, 6.0	
1M NaCl soluble colloids	4	Concentrate	84.4	(80.6–90.0)	6.4	(5.2–7.6)	4.9	(3.1–6.5)	7.3	(5.8–8.6)	6.4	(3.7–8.6)
1M NaCl insoluble colloid	2	Alfalfa	.....		3.6, 6.2		3.8, 4.1		.....		5.3, 5.5	
1M NaCl insoluble colloid	4	Concentrate	.....		3.7	(2.4–4.4)	1.9	(1.4–3.3)	5.7, 6.6		3.9	(2.5–5.7)

trate "calculi-provoking" diet described in a previous report [5] or alfalfa pellets, the greatest concentrations of proteins migrated similarly to that of the serum *alpha*-1 and *alpha*-2 globulins and albumin. Any increases in the concentration of *alpha*-globulin in urine would increase the concentration of urinary protein-bound carbohydrate since the majority of serum glycoproteins are present in the *alpha*-globulin fraction.

Pooled salt-insoluble "uromucoid" fraction and serum protein were treated at pH 12.0 as described in the section on materials and methods and were subjected to electrophoretic analysis at pH 12.0 in 0.1M glycine buffer as previously performed by Boyce *et al.*[2] on human "uromucoid." Boyce *et al.*[2] reported that this highly soluble form had lost nearly two thirds of its original hexosamine content. The majority of the ovine salt-insoluble "uromucoid" fraction treated in this manner separated as one component as reported previously in man.[2] The ovine serum protein, however, contained a small component corresponding to the mobility of this "uromu-

TABLE 3—Changes in the Composition and Concentration of the Phosphotungstic Acid—Precipitable Fraction* from Urine of Wethers Following Dialysis and Ultrafiltration**

Sample No.	24-hr. urine volume (ml.)	Treatment	(1) Tyrosine equivalents *mg./100 ml. urine*	(2) Protein*** conc. *mg./100 ml. urine*	(3) Hexosamine conc. *mg./100 ml. urine*	Ratio (2)/(3)
1	2,410	None	5.39	61.5	0.81	76
		Dialyzed †	0.596	6.8	0.36	19
		Ultrafiltered	0.026	0.3	....‡	....
2	1,380	None	13.10	150.4	1.06	142
		Dialyzed	0.982	11.0	0.83	13
		Ultrafiltered	0.105	1.2	....‡	....
3	2,520	None	5.87	67.0	1.15	58
		Dialyzed	0.807	9.2	0.86	11
		Ultrafiltered	0.026	0.3	....‡	....
4	2,595	None	3.0	34.2	1.2	28
		Dialyzed	0.429	4.9	0.53	9.3
		Ultrafiltered	0.026	0.3	....‡	....
5	1,620	None	5.73	65.4	1.93	34
		Dialyzed	1.62	18.5	0.86	21
		Ultrafiltered	0.026	0.3	....‡	....

* All aliquots were precipitated with phosphotungstic acid as recommended by Winzler [16] following initial adjustment of pH to 6.8 and the removal of the heat-coagulable proteins with perchloric acid.

** Experimental "calculi-provoking" diet described previously.[5]

*** Protein values calculated assuming a composition of 9% tyrosine.

† Dialyzed against ion-free water until specific conductivity less than 10 micromhos/cm. (approximately 48–72 hours).

‡ Concentration too low for measurement.

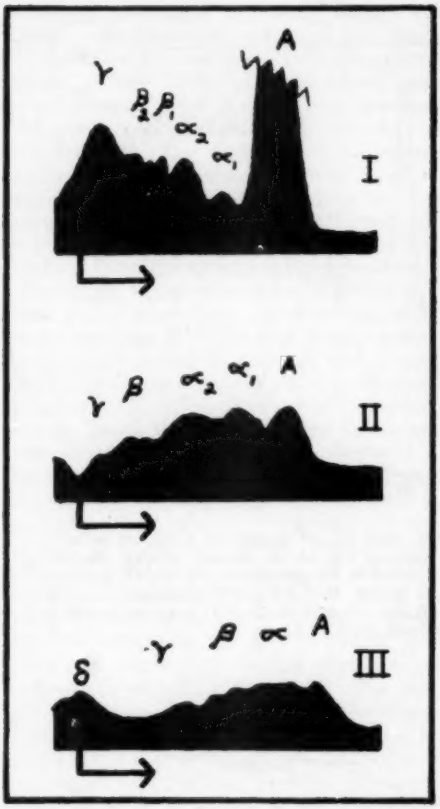


Fig. 1—Tracings of comparative electrophoretic patterns, at pH 8.6, of ovine serum proteins (I) and pooled 1M NaCl soluble urinary colloids from sheep fed a "calculi-provoking" diet[5] (II) and from sheep on a diet of alfalfa pellets (III). Veronal buffer, pH 8.6, ionic strength 0.1, 180 minutes, descending toward anode.

coid" fraction, in contrast to the findings in man. The electrophoretic separations of the "uromucoid" fraction from wethers fed either alfalfa or concentrate rations[5] were identical in presenting (Fig. 2) one major component and two smaller components with greater mobilities. The two components of lesser concentration could well be degradation products resulting from a different degree of hydrolysis of an originally homogeneous glycoprotein at pH 12.0.

Serum proteins from 7 sheep were separated on DEAE-C as described in the section on materials and methods. Serum protein effluent diagrams of wethers 882 and 889 are presented (Fig. 3, 4). In general, the pattern of elution of proteins closely resembled that reported for other mammals by Sober *et al.*[11] Effluent fractions were pooled, dialyzed, and lyophilized. The lyophilized residues were suspended in 0.1M veronal buffer and examined by paper electrophoresis. In most serum samples examined, pure *gamma*-globulin fractions were observed immediately in the first few tubes (Fig. 4-A, B) of effluent, suggesting little affinity of this fraction for the resin at the starting conditions. Following the introduction of buffer II, *beta* and *alpha* globulins usually appeared along with trace amounts of *gamma* globulins (Fig. 4-C). The majority of the *alpha* globulins appeared in the effluent following buffer III but were present in small concentrations throughout all fractions collected thereafter. Albumin appeared following the in-

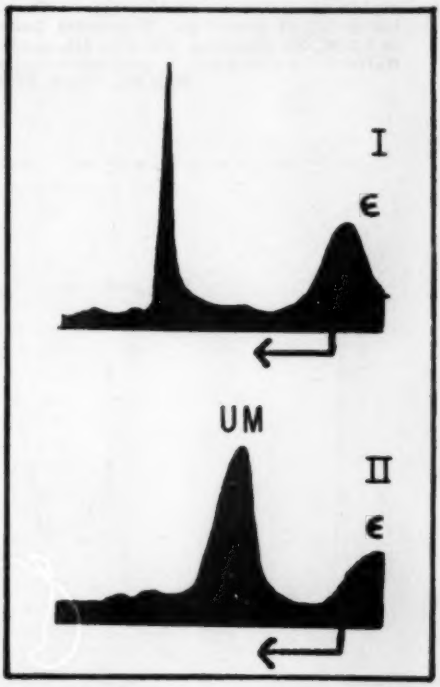


Fig. 2—Tracings of comparative electrophoretic patterns at pH 12.0 of normal ovine serum proteins (I) and the 1M NaCl insoluble uromucoid (UM) fraction treated at pH 12.0. Glycine buffer, pH 12.0, ionic strength 0.1, 130 minutes, ascending toward anode.

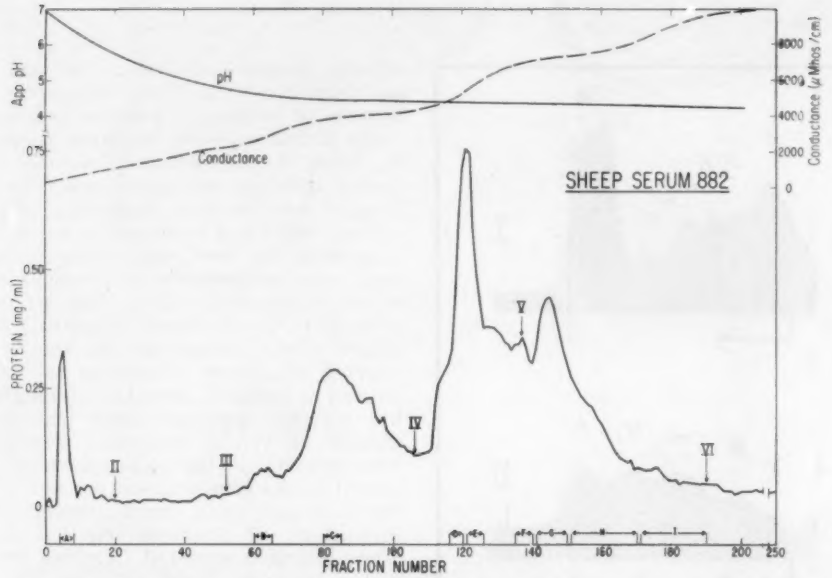


Fig. 3—Effluent diagram of serum from sheep 882: 280 mg. of protein in 4 ml. of buffer I applied to 25 Gm. of DEAE-cellulose. Fraction volumes 6.0 ml. of effluent, mixing chamber volume 125 ml. under 2 p.s.i. N_2 pressure. Buffers: I, 0.005 M Na phosphate, pH 7.0; II, gradient to 0.02M Na phosphate, pH 6.0; III, gradient to 0.05M NaH_2PO_4; IV, gradient to 0.05M NaH_2PO_4 + 0.02M NaCl; V, gradient to 0.05M NaH_2PO_4 + 0.05M NaCl; VI, gradient to 0.05M NaH_2PO_4 + 0.1M NaCl. Column, 35.0 by 2.5 cm.

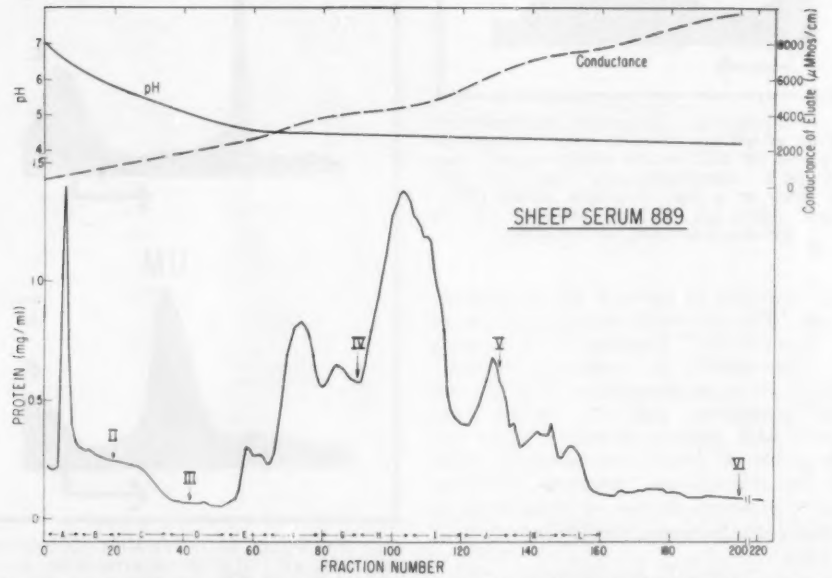


Fig. 4—Effluent diagram of serum from wether 889: 567 mg. of dialyzed and lyophilized serum proteins applied in 9 ml. of buffer I to 25 Gm. of DEAE-cellulose. Elution schedule as in Figure 3. Fractions, 9 ml.

troduction of buffer III and was in its highest concentration following the start of the buffer IV gradient. In no fractions except in the initial effluent which contained *gamma* globulin alone were *gamma*, *beta*, and *alpha* globulins and albumin totally resolved. The majority of the serum proteins, however, emerged in the order of their decreasing isoelectric points in the decreasing pH and increasing salt gradient. Quantitative serum protein recovery was obtained from the anion-exchange columns.

Seventeen sheep were tested * for soluble blood group substances. Twelve were type O; 5 were type R.[12] Similar reacting substances were found in the salt-soluble colloid isolated from the urine and in all cases agreed with the type observed in the blood. Soluble blood group substances in serum proteins eluted routinely from DEAE-C following the introduction of buffer V and were found in only fractions K and L as shown (Fig. 4). All fractions of the urinary salt-soluble colloid, eluted from DEAE-C, reacted positively for the presence of blood group substances.

Six pooled salt-soluble urinary biocolloid fractions were applied to DEAE-C columns and eluted with buffer gradients as with serum proteins (Fig. 5, 6). The pattern of protein elution resembled the serum proteins qualitatively, except that paper electrophoretic resolution of components was poorer and residual proteins routinely remained on the column following buffer gradient III. This urinary protein fraction (Fig. 5, 6G) was routinely removed with 1M KCl and its electrophoretic mobility was similar to that of serum *alpha*-globulin. This fraction may not represent a homogeneous protein but only a group of denatured proteins "trailing" in the elution pattern. Electrophoretic mobilities of the urine salt-soluble biocolloids from pooled effluents (Fig. 5, 6A-F) were in most fractionations as follows: A and B, pure *gamma* globulin; C, composed primarily of *alpha* globulin and albumin with small amounts of *gamma* and *beta* globulins; D, mainly *alpha* with traces of *gamma* and *beta* globulin; E and F, small amounts of equal proportions of *gamma*, *beta*, and *alpha* globulins. Quantitatively, it was quite apparent that the largest fractions of the urinary salt-soluble colloids were *alpha* globulins as observed by electrophoresis (Fig. 1, 2). Similar observations have been reported in man by others.[5, 16]

The pooled paper-filterable salt-insoluble "uromucoid" fraction, following alkaline degradation to the soluble state, reacted as a homogeneous protein in having little affinity for the resin at a high salt concentration near neutrality (Fig. 7). The treated "uromucoid" fraction under a different elution schedule eluted essentially as one major component following a very low concentration of Na phosphate at pH 7.7 (Fig. 8). Pooled insoluble fractions from wethers on alfalfa diets with or without diethylstilbestrol implantation eluted identically from DEAE-C resins.

Discussion

In previous experiments in which an increase in the concentration of a benzoate-adsorbed urinary glycoprotein fraction in sheep on a "calculi-provoking" diet was observed, a high percentage of the wethers died from urethral occlusion, rupture of the urinary bladder, and subsequent uremia.[5] The diet developed for the present biocolloid study was similar to existing feedlot rations in California and would not produce a high morbidity due to urolithiasis. The plan was to study the effects of an average concentrate diet on the levels of the total nondialyzable urinary biocolloids rather than with only one fraction. It cannot be assumed that the urinary biocolloids would necessarily be qualitatively similar on the non-calculi-provoking concentrate ration as compared to the calculi-provoking concentrate ration previously employed.[5] The pelleted diet used in the present study, however, has produced calculi in some California feedlot cattle.

It was quite evident in trial I of the present study that the concentration of 1M NaCl-soluble urinary colloid increased when wethers were placed on a pelleted concentrate diet. The reduction in urine volume in changing from the alfalfa to concentrate pellets most likely accounted for the increase in colloid concentration[8]; however, the age effect of animals being 6 weeks older cannot be entirely overlooked. Significant concentrations of principally *alpha*-glycoglobulins and albumin in the urine during concentrate feeding would certainly agree well with previous histochemical findings at this laboratory of a neutral glycoprotein matrix in ovine cal-

* The authors thank Dr. Clyde Stormont for the testing of soluble blood group substances in the plasma and urine.

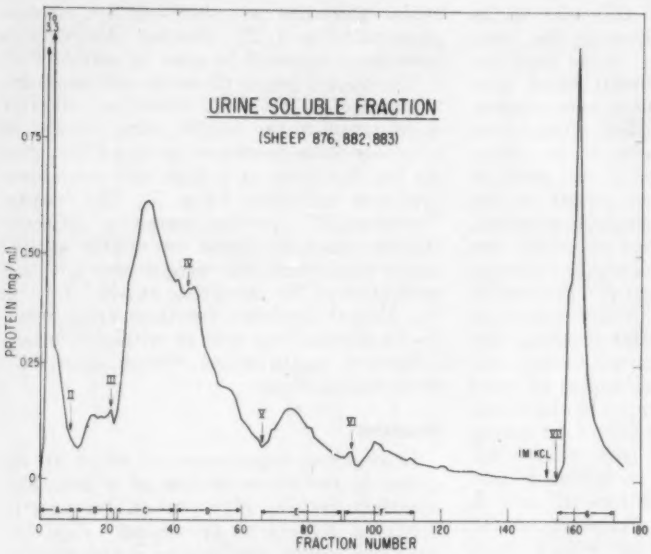


Fig. 5—Effluent diagram of salt (1M NaCl) soluble pooled urinary colloids from 3 wethers: 211 mg. of lyophilized colloids applied to column (8.5 by 1.0 cm.) of DEAE-cellulose. Buffer volumes were approximately 25% of those employed on serum proteins. Buffer concentrations were identical to serum chromatography gradient. Buffer VII, to 1M KCl. Fractions, 9 ml.

culous material. The observed increase in the blood seromucoid level [5] on the concentrate diet is also of interest, since this globulin fraction is mainly composed of neutral glycoproteins of smaller molecular size than most other globulin components.

It was of special interest that no statistically significant increase was observed in the salt-soluble urinary colloid per 24 hours per kilogram of body weight or in the insoluble fractions. Since the urinary colloids were filtered through coarse filter paper, a distinct possibility exists that some highly polymerized insoluble colloids could have been occluded in the paper along with the cells from the urinary system. Preliminary studies with five urine samples which were either centrifuged or filtered indicated that no salt-soluble colloid is trapped in paper filtration, but salt-insoluble colloid may be lost in certain samples.

In trials II and III, in which 15 and 60 mg. of diethylstilbestrol were implanted, extremely high levels of salt-soluble colloid occurred in approximately 50% of the sheep during each collection period. The lower levels in the other sheep were not due to a lack of a physiologic response to the hormone, since different sheep of both groups varied in their level of salt-soluble urinary colloid from trial II to III. Since urine from each sheep was collected only once in each trial, daily variations may have occurred. The amount of time required for ultrafiltration of each sample precluded the possibility of more collec-

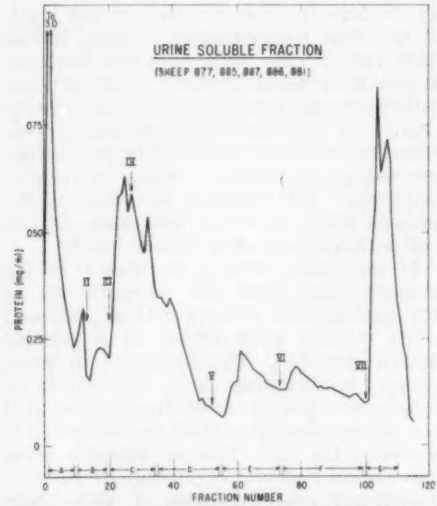


Fig. 6—Effluent diagram of salt (1M NaCl) soluble pooled urinary colloids from 5 wethers: approximately 280 mg. of lyophilized colloids to column (6.0 by 0.9 cm.) of adsorbent. Fractions, 9 ml. Elution buffers were identical to those employed in serum protein chromatography and in Figure 5.

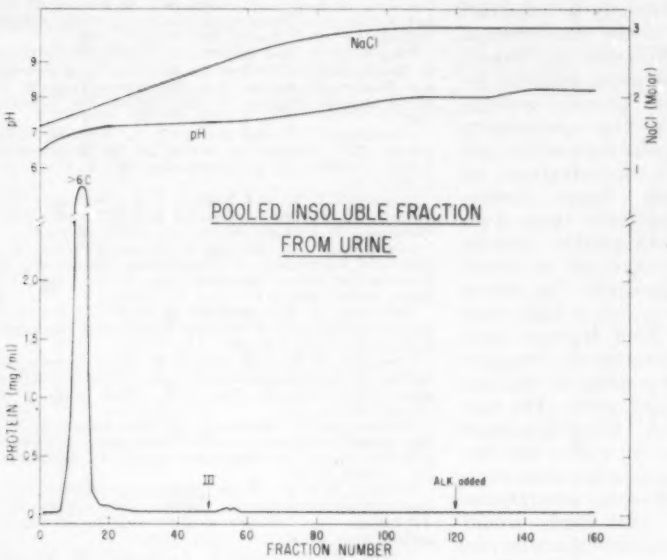


Fig. 7—Effluent diagram of salt (1M NaCl) insoluble pooled urinary fraction, uromucoid, from 11 wethers: 87 mg. of colloid treated at pH 12.0 in glycine buffer for 72 hours and dialyzed against buffer I. Fraction volume, 4 ml. Buffers: I, 0.005M Na phosphate-1.6M NaCl, pH 6.5 (in mixing chamber) 1,800 ml.; II, 6M NaCl in 0.05 M tris buffer, pH 7.8 (in reservoir at start of run); III, gradient to 6M NaCl in 0.05M tris buffer, pH 9.5.

tions from each sheep. These findings agree with those of Udall*; *i.e.*, a urinary protein-bound hexosamine fraction was elevated in sheep following estrogen implantation. The "calculi-provoking" effect of stilbestrol administration in sheep[15] has been reported. The exact identity of the biocolloids increasing in concentration following estrogen implantation is not known and awaits further study. It was of interest, however, that for two samples with an elevation of the 24-hour salt-soluble urinary colloid fraction from stilbestrol-treated wethers there were profiles of effluent diagrams on DEAE-C that were indistinguishable from those examined from control sheep on alfalfa hay.

* Udall, R. H.; Colorado State University, Fort Collins: Personal communication, 1958.

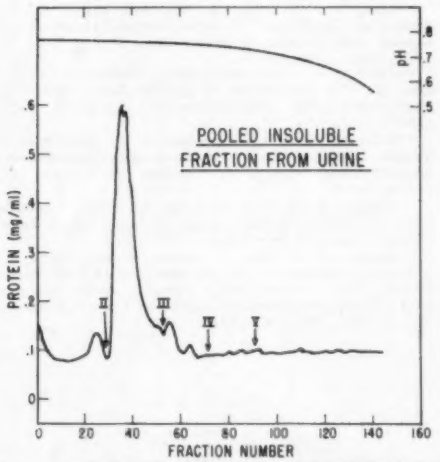


Fig. 8—Effluent diagram of salt (1M NaCl) insoluble pooled urinary fraction, uromucoid, from 6 wethers: 26 mg. of colloid treated at pH 12.0 in glycine buffer for 72 hours. Fraction volume, 6 ml. Buffers: I, distilled water; II, gradient to 0.0015M Na phosphate, pH 7.7; III, gradient to 0.05M Na phosphate-0.3M NaCl, pH 7.6; IV, gradient to 0.5M Na phosphate-1.0M NaCl; V, gradient to 0.05M Na phosphate-1.7M NaCl. Fraction volume, 4 ml.

Electrophoretic analysis of the salt-soluble urinary colloid was similar qualitatively from sheep on either alfalfa or concentrate rations with the majority of proteins migrating as *alpha* globulins and albumin, but with lesser amounts migrating as *gamma* and *beta* globulins. It was quite evident that the increase observed in the concentration of salt-soluble urinary colloids on the concentrate diet would be in actuality an increase in the *alpha*-glycoglobulin fraction which is known to contain conjugated proteins of a similar composition to the neutral glycoprotein matrix previously studied[3,4] from ovine calculous material. The salt-insoluble "uromucoid" fraction migrated electrophoretically at pH 12.0 as essentially one homogeneous

protein as reported previously in humans.[6]

Preliminary studies on the fractionation of ovine urinary biocolloids on DEAE-C agree with the electrophoretic analyses reported here. Again, the greatest concentration of salt-soluble protein components eluted similar to serum *alpha*-globulins and albumin with smaller concentrations of other protein fractions. Paper electrophoresis further substantiated these similarities. A urinary salt-soluble protein fraction in all samples could not be eluted using buffer gradients suitable for serum proteins and required salt at a high ionic strength for elution. This fraction may represent denatured components originating from the serum or a protein fraction originating in the urinary tract. The majority of the "uromucoid" fraction treated at pH 12.0 in 0.1M glycine buffer also appeared essentially as one homogeneous component in the electrophoretic separations. Evidence presented in this study would suggest that this salt-insoluble fraction can be purified readily on DEAE-C resins following partial degradation with loss of certain carbohydrate moieties at pH 12.0, and this "uromucoid" appears to be essentially one homogeneous protein when recovered from the urine of sheep. The major component separating from the "uromucoid" fraction in sheep may be similar to the virus inhibitory glycoprotein first isolated by Tamm and Horsfall in man.[13] Boyce *et al.*[1] have reported increased amounts of salt-insoluble "uromucoid" in human patients with calculous disease. Evidence presented in this study does not suggest that increased levels of a paper-filterable "uromucoid" fraction are associated with concentrate feeding in sheep.

References

[1] Boyce, W. H., Garvey, F. K., and Norfleet, C. M.: Proteins and Other Biocolloids of Urine in Health and Calculous Disease. I. Electrophoretic Studies at pH 4.5 and 8.6 of those Components Soluble in Molar Sodium Chloride. J. Clin. Invest., 33, (1954): 1287.

[2] Boyce, W. H., and Swanson, M.: Biocolloids of Urine in Health and in Calculus Disease. II. Electrophoretic and Biochemical Studies of a Mucoprotein Insoluble in Molar Sodium Chloride. J. Clin. Invest., 34, (1955): 1581.

[3] Cornelius, C. E., and Bishop, J. A.: Ruminant Urolithiasis III. Comparative Studies on the Structure of Urinary Concretions in Several Species. J. Urol., 85, (1961): 842.

[4] Cornelius, C. E., and Moulton, J. E.: Ruminant Urolithiasis: II. Histochemical Studies in Experimental Ovine Calculosis. J. Urology, 84, (Aug., 1960): 223–227.

[5] Cornelius, C. E., Moulton, J. E., and McGowan, B.: Ruminant Urolithiasis: I. Preliminary Observations in Experimental Ovine Calculosis. Am. J. Vet. Res., 20, (Sept. 1959): 863–871.

[6] Gorbman, A: Ultrafiltration of Urine for Collection and Biological Assay of Excreted Hypophyseal Hormones. Endocrinology, 37, (1945): 177.

[7] Johnston, G. W., and Gibson, R. B.: A Quantitative Method for the Determination of Total Protein in Cerebrospinal Fluid. Am. J. Clin. Path. (Tech. Suppl.), 8, (1958): 22.

[8] Newsom, I. E., Toluska, J. W., and Osland, H. B.: The Effects of Rations on the Production of Urinary Calculi in Sheep. Colorado Agric. Exptl. Sta. Tech. Bull. 311, (1943): 1–42.

[9] Philpot, J. S. L.: Direct Photography of Ultracentrifuge Sedimentation Curves. Nature, 141, (1938): 283.

[10] Schloss, B.: Colorimetric Determination of Glucosamine. Anal. Chem., 23, (1951): 1321.

[11] Sober, H. A., Gutter, F. J., Wyckoff, M. M., and Peterson, E. A.: Chromatography of Proteins. II. Fractionation of Serum Protein on Anion-Exchange Cellulose. J. Am. Chem. Soc., 78, (1956): 756.

[12] Stormont, Clyde: An Example of a Recessive Blood Group in Sheep. Genetics, 37, (1952): 577.

[13] Tamm, I., and Horsfall, F. L., Jr.: A Mucoprotein Derived from Human Urine which Reacts with Influenza, Mumps, and Newcastle Disease Viruses. J. Exptl. Med., 95, (1952): 71.

[14] Udall, R. H., Deem, A. W., and Maag, D. D.: Studies on Urolithiasis. I. Experimental Production Associated with Feeding in Steers. Am. J. Vet. Res., 19, (Oct., 1958): 825–829.

[15] Udall, R. H., and Jensen, Rue: Studies in Urolithiasis: II. The Occurrence in Feedlot Lambs Following Implantation with Diethylstilbestrol. J.A.V.M.A., 133, (1958): 514.

[16] Webb, T., Rose, B., and Sehon, A. H.: Biocolloids in Normal Human Urine. I. Amount and Electrophoretic Characteristics. Canad. J. Biochem. & Physiol., 36, (1958): 1159.

[17] Winzler, R. J.: Determination of Serum Glycoproteins. In *Methods of Biochemical Analysis*. Vol. 2, Interscience Publishers, New York, (1955): 279.

[18] Wolfson, W. Q., Cohn, C., Calvary, E., and Ichiba, F.: A Rapid Procedure for the Determination of Total Serum Protein, Total Protein, Total Globulin, *Alpha* Globulin, *Beta* Globulin and *Gamma* Globulin in 1.6 ml. of Serum. Am. J. Clin. Path., 18, (1958): 723.

SUMMARIO IN INTERLINGUA

Urolithiasis de Ruminantes. IV. Studios de Ultrafiltration, Electrophorese, e Chromatographia in Biocolloides Urinari de Oves

Le total nondialysabile biocolloides urinari de oves esseva isolate per technicas de ultrafiltration e separate in le duo fractiones de solubile e non-solubile in 1 M NaCl. Post que arietes habeva essite transferite ab un dieta de bullettas de lucerna ad un dieta de bullettas de concentrato, le nivellos urinari de biocolloides solubile in NaCl montava in parallela con un declino del volumine de urina. Nulle significative variationes occurreva in le excretion in 24 horas de biocolloide solubile in sal quando mesurate in unitates per kilogramma de peso

corporee, e nulle tal variationes occurreva in le filterabile fraction "uromucoide" non solubile in sal. Del altere latere, significative augmentos occurreva in le concentration del colloides urinari solubile in sal e in lor excretion diurne per kilogramma de peso corporee in oves que habeva recipite implantationes subcutanee de diethylstilbestrol. Le biocolloides urinari e le proteinas seral esseva fractionate con le uso de electrophorese e del technica a resinas de excambio anionic con cellulosa diethylaminoethylic.

The Creatinine Coefficient of Normal Sheep

M. Z. Barakat, Ph.D., and A. Abdalla, D.V.M.

SUMMARY

Normal sheep urine samples voided per day were collected in Egypt during the "green" and "dry" seasons from normal Ossimi and Rahmani mature rams which varied in age and body weight. Toluene was used as a preservative for each 24-hour urine sample.

The daily output of urinary creatinine and creatine was determined by Folin's method for these two breeds. The total excretion of urinary creatinine plus creatine per day was found to be fairly constant for individual sheep.

The creatinine coefficient was established by adding the creatinine nitrogen to the creatine nitrogen output per day and dividing the total, expressed in milligrams, by the body weight of sheep (in kilograms). The mean creatinine coefficients of Ossimi and Rahmani sheep were found to be 31.02 and 32.58, respectively. The creatinine coefficient decreased with increased age and body weight.

INTRODUCTION

There appears to be no report in the literature concerning the creatinine coefficient of normal sheep. Only scattered data have been reported on the analysis of random specimens of sheep urine.[5] Although the percentage distribution of nitrogen in the urine of fasting sheep [2] is known, the results are derived from the analysis of random samples and are not an actual representation of the daily creatinine and creatine output in normal sheep urine.

Experimental Procedures

This investigation was part of a general scheme for analysis of normal sheep urine. The rams used belonged to the Biochemistry Department, Faculty of Veterinary Medicine, Cairo University. They were kept under hygienic conditions. The ration varied according to the time of sample collection, but was considered normal and was adequate to maintain normal metabolic requirements. During the "green" season, between February and May, the daily ration contained berseem, *Trifolium alexandrinum* (12 lb.), and wheat bran (2 lb.); during the "dry" season, between June and August, it contained wheat bran (2 lb.) and barley (3 lb.). Wheat straw was provided ad libitum as roughage. Rice straw was used as bedding, and the animals usually ate some of it. Water was supplied ad libitum.

A 24-hour urine sample was collected by means of an apparatus (Fig. 1, 2) which was, in its main features, analogous to that devised by Bassett.[1] This method seemed to be the most practical and satisfactory for collecting urinary output per day without any loss. The urine specimens were collected from 26 Ossimi and 26 Rahmani mature rams which varied in age (from 1 year 6 months to 2 years 6 months, and from 1 year 3 months to 2 years 10 months, respectively) and body weight (from 32.35 to 53.92 kg., and 26.96 to 62.90 kg., respectively).

A total of 150 samples of normal sheep urine were collected from the 52 sheep (two breeds) for determining creatinine and creatine. Of 71 samples from Ossimi sheep, 31 and 40 urine samples were collected during the "green" and "dry" seasons, respectively. Of 79 samples from Rahmani sheep, 38 and 41 were collected during the respective seasons. One to three samples were taken from each animal. To each sample was added 5 ml. of toluene as a preservative.

The determination of creatinine and creatine in each 24-hour urine sample was carried out by Folin's method.[3]

Results and Discussion

An analysis of the results of the quantitative estimation of creatinine and creatine is shown (Table 1), as is the daily total excretion of creatinine and creatine (Table 2). The creatinine coefficient [10] was constructed by adding the creatinine nitrogen to the creatine nitrogen output per day and dividing the total, expressed in milligrams, by the body weight of the sheep, expressed in kilograms (Table 3).

The relationship of the creatinine coefficient to age and body weight is shown (Table 4).

The mean creatinine output varied from

Received for publication April 24, 1961.

From the Biochemistry Department, Faculty of Veterinary Medicine, Cairo University, Giza, Cairo, U.A.R.

TABLE 1—Creatinine and Creatine (Gm./Liter) in 26 Ossimi and 26 Rahmani Sheep

Breed	Creatinine				Creatine			
	Max.	Min.	Mean	S.E.	Max.	Min.	Mean	S.E.
Ossimi	1.25	0.20	0.76	± 0.0335	4.79	2.07	3.16	± 0.0829
Rahmani	1.43	0.31	0.81	± 0.0036	4.68	1.80	3.29	± 0.0724

TABLE 2—Total Daily Output (in Grams) of Creatinine and Creatine in 8 Ossimi and 8 Rahmani Sheep

	Ossimi			Rahmani		
	Creatinine	Creatine	Total	Creatinine	Creatine	Total
Max.	0.94	3.35	3.60	1.14	3.22	3.51
Min.	0.25	2.66	3.59	0.28	2.45	3.46
Mean	0.70	2.89	3.60	0.78	2.72	3.50
S.E.	± 0.04	± 0.04	± 0.00	± 0.04	± 0.08	± 0.00

to be associated with increased catabolism of protein, the liberation of creatine being more rapid than its conversion to creatinine.

For individual sheep, it appears that the creatine output rises and falls inversely with the creatinine output in a 24-hour period, with the result that the total daily excretion of creatinine and creatine in nor-

TABLE 3—Creatinine Coefficient of Normal Sheep (26 Ossimi and 26 Rahmani)

Ossimi breed				Rahmani breed			
Max.	Min.	Mean	S.E.	Max.	Min.	Mean	S.E.
36.81	22.51	31.02	± 0.7484	42.93	20.44	32.58	± 0.9752

0.81 to 0.76 Gm. per liter (Table 1). The daily creatinine output in normal sheep urine varied from about 1.21 to 0.23 Gm., with an average of 0.73 Gm. per day. Creatinine excretion is practically independent of the dietary protein and is not influenced by ordinary exercise [7] or the urine volume.[8] Creatinine is determined chiefly by the size of the body and muscular development.[4, 6, 9] Creatinine appears to be entirely a waste product not utilized by the body, and its conversion to creatine in the body does not occur.

It appears that creatine is a normal constituent of sheep urine, with a mean value of 3.16 Gm. per liter of urine in the Ossimi breed and 3.29 Gm. in Rahmani sheep. The creatine content of normal sheep urine varies from 3.80 Gm. to 2.24 Gm., with an average of 3.02 Gm. per day. This is not unexpected, since creatine has been reported in the urine of fasting sheep and of the nonfasting cow.[2] Creatinuria appears

TABLE 4—Relationship of Creatinine Coefficient to Age and Body Weight of Sheep (18 Ossimi and 18 Rahmani)

Breed	Age		Body weight (kg.)	Creatinine coefficient
	(Yr.)	(Mo.)		
Ossimi	1	6	32.35	36.21
Rahmani	1	10	36.84	35.11
Ossimi	2	6	53.92	23.51
Rahmani	2	10	62.90	21.16

Fig. 1—The collecting chamber, showing back-flow prevention valve (x).

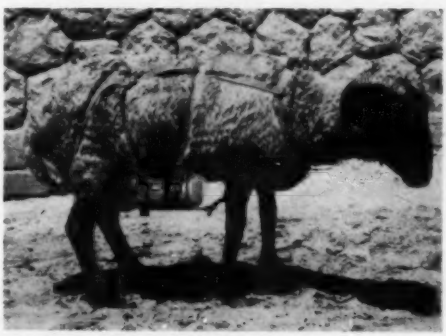


Fig. 2—The urine-collecting apparatus in position on a ram.

mal sheep urine is fairly constant for an individual animal (Table 2).

From the results, it seems that the mean creatinine coefficient (Table 3) is approximately the same for both breeds: 31.02 for the Ossimi sheep and 32.58 for the Rahmani sheep.

The creatinine coefficient decreases with increase in age and, consequently, with an increase in body weight (Table 4). Since the bulk (98%) of the creatine of the body is in the muscles, there is a rough correlation between the creatinine-creatine excretion and the amount of muscular tissue in the body.

References

[1] Bassett, E. G.: Apparatus for Continuous Collection of Urine from the Grazing Ewe. New Zealand J. Sci., 34, (1952): 76.

[2] Dukes, H. H.: *The Physiology of Domestic Animals.* 7th ed. Baillière, Tindall & Cox, London (1955): 504–505.

[3] Folin, O.: The Determination of Creatinine and Creatine. J. Biol. Chem., 17, (1914): 469.

[4] Harding, V. J., and Gaebler, O. H.: The Constancy of the Creatine-Creatinine Excretion in Children on a High Protein Diet. J. Biol. Chem., 54, (1922): 579.

[5] Healy, D. J., Bullard, J. F., and Spears, H. D.: A Normal Sheep Urinalysis. J.A.V.M.A., 73, (May, 1928): 87.

[6] Hodgson, P., and Lewis, H. B.: Physical Development and the Excretion of Creatine and Creatinine by Women. Am. J. Physiol., 87, (1928): 288.

[7] Hunter, A.: The Physiology of Creatine and Creatinine. Physiol. Rev., 2, (1922): 586.

[8] Marshall, E. K., Jr.: The Influence of Diuresis on the Elimination of Urea, Creatinine and Chlorides. J. Pharm. & Expt. Therap., 16, (1921): 141.

[9] Shaffer, P. A.: The Excretion of Kreatinin and Kreatin in Health and Disease. Am. J. Physiol., 23, (1908–09): 1.

[10] Varley, H.: *Practical Clinical Biochemistry.* 1st ed. Medical Books Ltd., London (1954): 146.

Summario in Interlingua

Le Coefficiente de Creatinina in Oves Normal

Specimens de normal urina ovin, representante le vacuation total de 24 horas, esseva colligite in Egypto durante le saisones "verde" e "sic" ab matur arietes Ossimi e Rahmani de varie etates e pesos corporee. Tolueno esseva usate como agente de preservation.

Le rendimento diurne de creatinina e creatina urinari in le duo mentionate racias esseva determinate secundo le methodo de Folin. Esseva trovate que le combinate excretion diurne de creatinina e creatina es satis constante in le ove individual.

Le coefficiente de creatinina esseva establite per adder le nitrogeno de creatinina al nitrogeno de creatina e divider le total (exprimite in milligrammas) per le peso corporee del ove (exprimite in kilogrammas). Le coefficiente medie de creatinina in oves Ossimi e Rahmani esseva determinate como 31,02 e 32,58, respectivemente. Le coefficiente de creatinina declinava con le avantiamento del etate e le augmento del peso.

Factors Influencing the Incidence of Chronic Pneumonia in Lambs

André Lagacé, D.V.M., Ph.D.; W. D. Pounden, D.V.M., Ph.D.; D. S. Bell, M.S.; K. D. Weide, D.V.M., M.S.

SUMMARY

An attempt was made to study the influence of housing, management, and breed on the incidence of pneumonia in lambs. In experiment 1, four lots of lambs were housed either in closed or open-door pens, with or without brooders, and a fifth lot was subjected to close housing alternated with chilling.

Of the lambs in the chilled group, 27% had extensive pneumonic lesions when slaughtered compared with between 37 and 58% in the other four groups. However, early mortality was 33% in the former as opposed to but 9% in the other four groups. There was half as much pneumonia in lambs that developed well enough to be slaughtered in August as compared with lambs of similar age slaughtered in October.

In experiment 2, in lots 6 through 13, the association between breeds and infant mortality or pneumonia was not significant. Of the creep-fed lambs, 75% had pneumonic lesions while only 53% of the non-creep-fed lambs had similar lesions. There was a gradual ascending incidence of pneumonic lesions in lambs born from the first week of February to the first week of April.

INTRODUCTION

Previous work disclosed the presence of widespread subclinical chronic pneumonia in Ohio lambs.[6] A similar condition has also been reported in California.[4,5] *Pasteurella* organisms, pleuropneumonia-like organisms, and a viral agent were found to be associated with these pneumonic lesions.[2,3]

While pneumonic lesions could not be produced experimentally in lambs with only one or two of these agents, the three agents used together produced lesions in 50% of the lambs. When an artificial stressor was used in combination with two of the infectious agents, pneumonic lesions were constant in all experimental animals.[1]

Experiment 1 was designed to study variations in housing and management and their influence on the incidence of chronic pneumonia in lambs at time of slaughter. Experiment 2 was designed to determine the relationship of breeds and creep-feeding to the incidence of pneumonia.

Materials and Methods

Experiment 1.—Lambs from the same flock were raised under five different environmental conditions. Ewes and lambs (16 lambs) in lot 1 were confined to a pen without electric lamp brooders. Ewes and lambs (36 lambs) in lot 2 were allowed daily exercise with an "open-door" outside lot exposure. Lamp brooders were used. Ewes and lambs (13 lambs) in lot 3 were confined to the pen, and lamp brooders were used. Ewes and lambs (47 lambs) in lot 4 had an "open-door" outside lot exposure were forced to take daily field exercise. No lamp brooders were used for this lot. Ewes and lambs (33 lambs) in lot 5 were confined to the pen and subjected to alternate heating and chilling with no brooders. This was accomplished by hanging canvas sidings from the ceiling on the two open sides of the pen to limit the space, reduce ventilation, and promote a high humidity-heating effect at night. Early each morning and during the rest of the day, the canvas sidings were removed and all doors and windows were opened to encourage drafts. Automatic temperature and humidity recorders were kept in these pens.

These lambs were all born between February 1 and March 7, except 1 or 2 lambs in each group.

Experiment 2.—A group of 223 lambs and their dams, which included all the sheep in one of our barns, were used in these studies. The ewes had been allotted to four pens according to breed. Ewes in each pen were subsequently allotted at random to two lots containing an equal number of ewes: Lots 6 and 7 contained purebred Columbias;

Received for publication Jan. 13, 1961.

From the Department of Veterinary Science (Lagacé, Pounden, and Weide) and the Department of Animal Science (Bell), Ohio Agricultural Experiment Station, Wooster, Ohio. Dr. Lagacé is now with the École de Médecine vétérinaire, St. Hyacinthe, Qué.

The authors thank Dr. C. R. Weaver, station statistician, for the assistance given in the statistical analysis of the data.

Fig. 1—Extensive pneumonic lesions in a lamb (right apical lobe).

lots 8 and 9, Hampshire-Columbias; lots 10 and 11, Southdown-Columbias; and lots 12 and 13, Merino crossbred. The creep-fed lambs (lots 7, 9, 11, 13) were given a mixture of whole corn and oats from March 15 until weaning time at 120 days of age.

All lambs dying before slaughter were necropsied, and pneumonic lesions were evaluated. Pneumonic lesions were likewise examined and evaluated in all lambs slaughtered at market age. Lesions were classified as extensive when at least one fourth of a lobe was affected (Fig. 1). Anything less than this was marked as slight.

Results

Experiment 1.—Extensive lesions of pneumonia in the lambs sent to slaughter varied from 27% in lot 5 to 58% in lot 2, while infant (lambs under 15 days of age) mortality varied from none in lot 3 to 33% in lot 5 (Table 1). With the exception of the 13 lambs in lot 3, the lots with the highest infant mortality had the lowest incidence of pneumonia. Deaths in lambs over 15 days of age were also fewer in groups with a high infant mortality. Twenty per cent of the deaths of lambs over 15 days of age were due to acute pneumonia. The incidence of deaths before 15 days of age was apparently not related to variations in ambient temperature (Fig. 2).

In lots 1 to 5, 57 lambs grew sufficiently to be slaughtered in August but the other 47 were not killed until October. In lambs born the same week, two to five times as much pneumonia was found in those slaughtered in October (Fig. 3).

Experiment 2.—Only 18% of the Merino-cross lambs had extensive lesions of pneumonia. Thirty-two per cent of the purebred Columbias as well as 50% and 48%, respectively, of the Hampshire-Columbias and the Southdown-Columbias had extensive lesions of pneumonia. The breeds with the highest infant mortality had the least pneumonia (Table 2). However, the *chi*-square test of association between breeds and pneumonia or infant mortality was not statistically significant. The lots with the creep-fed lambs (lots 7, 9, 11, and 13) were compared with the lots containing lambs not creep-fed (lots 6, 8, 10, 12). While the incidence of extensive lesions of pneumonia was similar in both groups (46 and 42%, respectively), the total incidence of pneumonia, including slight lesions, was 75% of the creep-fed lambs and only 53% of the lambs not creep-fed.

TABLE 2—Relationship of Breeds of Sheep to the Incidence of Pneumonia (Experiment 2)

Lots	No. of lambs	Pneumonia (%)	Deaths at less than 15 days of age (%)
6, 7—Purebred Columbia	67	32	20
8, 9—Hampshire-Columbia	73	50	12
10, 11—Southdown-Columbia	60	48	8
12, 13—Merino crossbred	23	18	17

TABLE 1—Influence of Housing and Management on Incidence of Chronic Pneumonia and Infant Mortality in Lambs (Experiment 1)

Treatment	Lot	No. of lambs	Extensive pneumonia at slaughter (%)	Deaths <15 days (%)	Deaths >15 days (%)
Confinement and no brooders	1	16	50	6	19
"Open-Door" and brooders	2	36	58	5	28
Confinement and brooders	3	13	39	0	0
"Open-Door" and exercise; no brooders	4	47	37	17	8
Close housing; sudden chill; no brooders	5	33	27	33	0

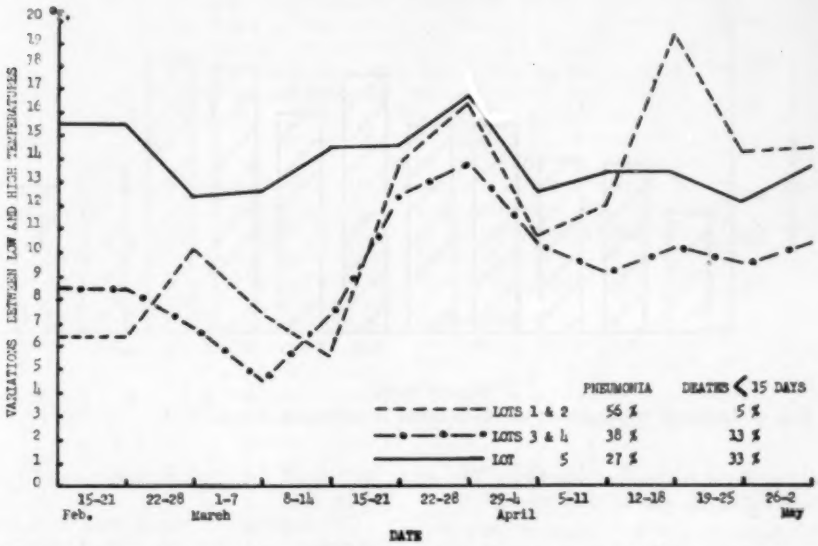


Fig. 2—Mean weekly variations between low and high temperature in different pens of lambs (experiment 1).

We analyzed statistically the relationship of *Muellerius capillaris* lesions, creep-feeding, and date of birth with the incidence of pneumonia. Pneumonia was assigned the rating 1, if absent; 2, if slight; 3, if extensive. The relationship of the other variables to the pneumonia rating was then investigated by the usual multiple regression techniques. Parasitic lung lesions turned out to be of no consequence and were omitted

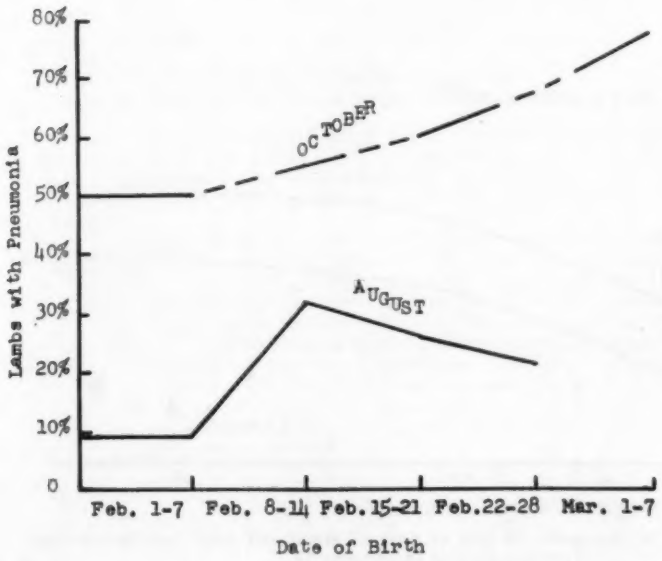


Fig. 3—Incidence of pneumonia lesions in lambs in relation to date of birth and month of slaughter (experiment 1).

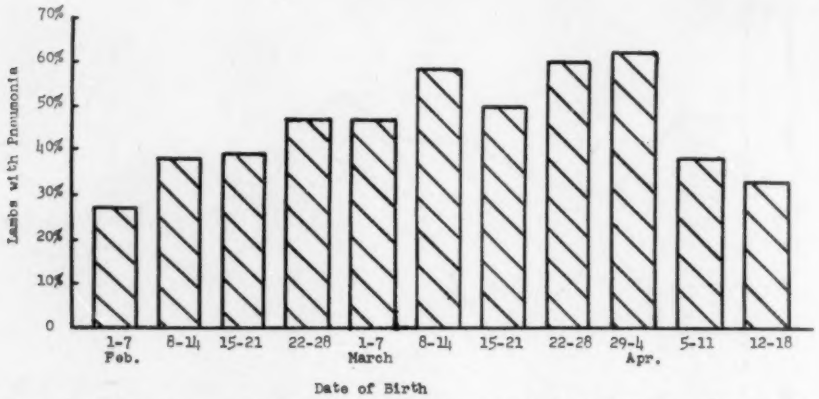


Fig. 4—Incidence of pneumonic lesions in lambs in relation to date of birth (experiments 1, 2).

from the final regression solution. The resulting equation was as follows:

$$P = 0.3452 + 0.3018\,C + 0.0344\,D - 0.0002\,D^2$$

The multiple correlation coefficient equalled 0.2412, which is highly significant. The regression coefficient for C (creep-feeding) was also highly significant, while those for D (date of birth) and D^2 were significant at probability 0.10 and 0.20, respectively. The equation was solved at six points within the range of the variables and plotted (Fig. 5).

The increase in pneumonia with time is, of course, not well established by these statistics; however, the trend is certainly strong.

Of the single lambs, 41% had pneumonic lesions; exactly the same percentage of twin lambs had similar lesions.

The date of birth was a factor in the incidence of extensive lesions of pneumonia

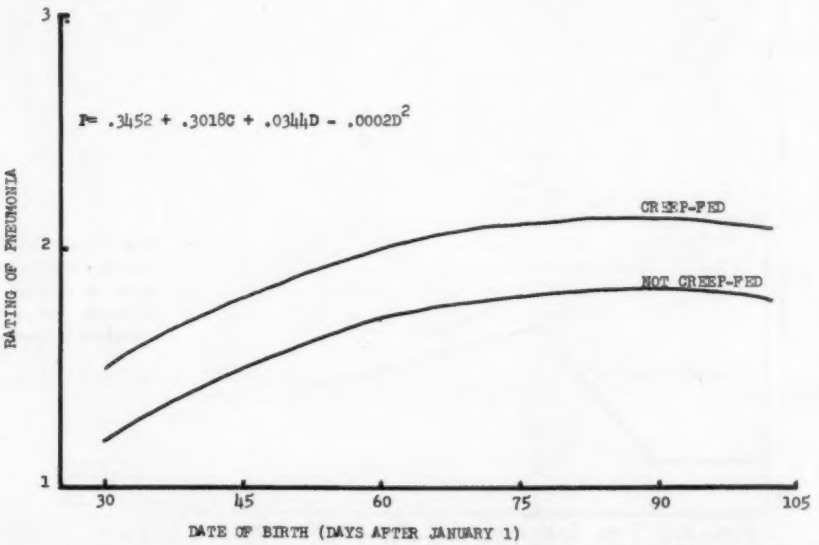


Fig. 5—Relationship to pneumonia, of date of birth of lambs and creep feeding—multiple regression analysis (experiment 2).

in the lambs used in these experiments. Only 27% of the lambs born in the first week of February had lesions of pneumonia, and the percentage gradually increased to a peak of 62% in the week of March 29 to April 4 (Fig. 4).

Discussion

The pen of lambs subjected to heating and chilling had the least pneumonia but the most infant mortality. This relation was also true of the other experimental groups. Apparently, the type of pneumonia dealt with in these studies became established especially in lambs weak at birth. When the experimental treatments produced death of the weaklings, they also reduced the incidence of pneumonia. Lambs of a given age which reached market condition earlier had less pneumonia. While infant mortality did not seem to be related to the variations in ambient temperature, it is possible that the cold drafts produced in lot 5 as well as the forced outside exercise in lot 4 were the primary reasons for the higher infant mortality in these two lots. Purebred Columbia and Merino crossbred lambs had less pneumonia than Hampshire and Southdown crossbred lambs but they also had more infant mortality. The higher incidence of pneumonia in creep-fed lambs is difficult to evaluate. The gradually ascending incidence of pneumonia in lambs born from the first week of February to the first week of April could possibly be related to a natural build-up in the number of animals which would increase the danger of infection for lambs born late in the season. In past years, it was noticeable that there was also an ascending incidence of pneumonia in groups of lambs slaughtered in August, September, October, and November, regardless of their date of birth.

References

[1] Hamdy, A. H., and Pounden, W. D.: Experimental Production of Pneumonia in Lambs. Am. J. Vet. Res., 20, (Jan., 1959): 78–83.

[2] Hamdy, A. H., Pounden, W. D., and Ferguson, L. C.: Microbial Agents Associated with Pneumonia in Slaughtered Lambs. Am. J. Vet. Res., 20, (Jan., 1959): 87–90.

[3] Hamdy, A. H., and Sanger, V. L.: Characteristics of a Virus Associated with Lamb Pneumonia. Am. J. Vet. Res., 20, (Jan., 1959): 84–86.

[4] McGowan, B., Moulton, J. E., and Shultz, G.: Pneumonia in California Lambs. J.A.V.M.A., 131, (Oct. 1, 1957): 318–323.

[5] McKercher, D. G., Biberstein, E. L., and Wada, E. M.: A Review of Recent Findings in Infectious Diseases of Sheep. I. Virus Diseases. J.A.V.M.A., 134, (June 15, 1959): 549–555.

[6] Pounden, W. D., Bell, D. S., Edgington, B. H., and Thomas, D. L.: Disease Conditions Observed in Lambs at Slaughter. J.A.V.M.A., 128, (March 15, 1956): 298–301.

SUMMARIO IN INTERLINGUA

Factores Que Exerce un Influentia Super le Incidentia de Chronic Pneumonia in Agnos

Esseva interprendite le tentativa de studiar le influentia que es exercite super le incidentia de pneumonia in agnos per le factores de stabulation, servicio general, e racia. In experimento 1, quatro gruppos de agnos esseva stabulate a portas clause o aperte, con o sin lampas de incubation, e un quinte gruppo esseva subjicite a stabulation clause in alternation con algoration.

Inter le agnos del gruppo algorate, 27% habeva extense lesiones pneumonic al tempore del abattimento, in comparation con inter 37 e 58% in le altere quarto gruppos. Tamen, le precoce mortalitate esseva 33% in le gruppo a algoration e solmente 9% in le altere quatro gruppos. Le incidentia de pneumonia in agnos satis ben disveloppate pro esser abattite in augusto esseva solmente un medietate de illo in agnos del mesme etate abattite in octobre.

In experimento 2, le observation de octo gruppos de agnos (gruppos 6 a 13) revelava nulle significative association inter racia e mortalitate o pneumonia infantil. Inter le agnos recipiente mais e avena, 75% habeva lesiones pneumonic. Inter le agnos non assi alimentate, solmente 53% habeva simile lesiones. Esseva constatate un augmento gradual del incidentia de lesiones de pneumonia in parallela al datas de nascentia ab le prime septimana de februario usque al prime septimana de april.

Microbiologic Survey of Pneumonic and Normal Swine Lungs

Conrad L'Ecuyer, D.V.M.; W. P. Switzer, D.V.M., Ph.D.; E. D. Roberts, D.V.M.

SUMMARY

The results of the examination of 86 pneumonic and 15 normal swine lungs for viruses, rickettsias, *Mycoplasma hyorhinis*, and bacteria are reported. One viral agent was recovered from the pneumonic lungs; none were recovered from the normal specimens. The viral agent was cytopathogenic for swine kidney cell cultures but, experimentally, it produced no lesions in swine. *Mycoplasma hyorhinis* was recovered from 51.1% of the pneumonic, and 6.6% of the normal lungs. *Pasteurella multocida* and *Streptococcus sp.* were the principal bacteria recovered from the pneumonic lungs, while only alpha-hemolytic streptococci appeared in significant numbers in the normal lungs. No rickettsias were recovered. Swine influenza virus was not isolated.

INTRODUCTION

This study was undertaken to determine the current microbiologic status of swine pneumonia. To achieve this objective, grossly normal and grossly pneumonic swine lungs were examined for bacteria, pleuropneumonia-like organisms, rickettsias, and viruses.

Review of Literature

Betts,[2] Hjärre,[15] and Young [44] have published general literature reviews on swine pneumonia. The bacterial aspects of the etiology of swine pneumonia have been reported in surveys of normal and pneumonic lungs conducted by Spray,[32] Thorp *et al.*,[40] Morcos *et al.*,[20] Rislakki,[28] Palyusik,[21] and Ryu.[25] The bacteria recovered by these workers are shown in Table 1.

Many bacterial species have been incriminated as primary agents of swine pneumonia, but only a few have been confirmed as agents experimentally. Sippel *et al.*,[31] and Ellis *et al.*,[9] recovered *Chromobacterium violaceum* from outbreaks of acute, frequently fatal pneumonia. Sippel *et al.*[31] used pure cultures to reproduce the condition by oral infection. Thal and Rutqvist [30] produced extensive pneumonic lesions in swine, with pure cultures of *Corynebacterium equi* recovered from natural cases. A *Hemophilus*-like organism was reported by Pattison *et al.*[22] to produce characteristic pneumonia and pleuritis when inoculated in combination with hog cholera virus. Later, Matthews and Pattison [19] identified a similar *Hemophilus*-like organism as a member of the *Hemophilus parainfluenzae* group and found that typical lesions could be produced if a sufficient number of organisms were inoculated.

Switzer [33] cultured *M. hyorhinis* from 20 of 28 pneumonic swine lungs and associated this organism with the serositis lesions which frequently accompany swine pneumonia. Carter and Schroder [4] recovered *Mycoplasma sp.* from the lungs of 14 swine, 6 of which had pneumonia and 8 of which had pneumonia complicated by serositis. Twenty-three normal packing plant swine lungs were free of these organisms. More recently, Carter and Schroder [5] demonstrated that intranasal instillation of swine *Mycoplasma sp.* did not produce pneumonia, whereas pneumonic lung suspensions or filtrates free of bacteria or *Mycoplasma sp.* produced typical lesions when administered by the same route. Forray [12] recovered *Mycoplasma sp.* from 3 of 20 animals dead of bronchopneumonia. Switzer [33] failed in attempts to produce pneumonic lesions in swine with pure cultures of *M. hyorhinis*.

Shope [29] has reviewed the literature on swine influenza. Recently, Bakos [1] obtained a definite cytopathic effect (CPE) with the Iowa 15 strain of swine influenza virus after four or five passages on primary swine kidney cell cultures, while a Wisconsin strain isolated by Kissling was only slightly cytopathic after 12 cell culture passages. Young and Underdahl [43] found serologic evidence of contact with swine or human influenza viruses in only 15% of over 2,000 midwestern swine.

In 1954, Sasahara *et al.*[25, 27, 28] reported an influenza-like disease of swine in Japan which produced severe illness and some mortality among young swine, but which produced no clinical signs in older animals. These investigators recovered a hemagglutinating virus from chicken embryos by inoculating pneumonic material by way of the

Received for publication Oct. 26, 1960.

From the Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University of Science and Technology, Ames.

Published as paper n.s. 618, Veterinary Medical Research Institute, Iowa State University. Portions of this work will be included in a thesis submitted to the Graduate School, Iowa State University, in partial fulfillment of the requirements for the Ph.D. degree. Dr. L'Ecuyer is an AVMA Research Fellow.

This work was supported in part by NC-13 funds.

The authors thank Dr. R. F. Ross and Mrs. Janet Welch for their assistance.

TABLE 1—Microorganisms Recovered from Normal and Pneumonic Swine Lungs

Investigator	Spray [23]		Thorp [40]		Morcos [20]		Rislakki [28]	Palyusik [21]	Ryu [25]
Country	United States		United States		Egypt		Finland	Hungary	United States
Source of specimens	Packing plant		Field		Field and packing plant		Packing plant	Packing plant	Field
Condition of lungs*	P	N	P	N	P	N	P	P	P
Total specimens	314	100	166	38	100	20	30	49	108
% Sterile	.....	.....	28	97	.....	.....	57	10	.....
Pasteurella	54	4	27	.....	55	...	17	.....	58
Streptococci	35	12	40	1	5	.....	17	+	40
Alcaligenes	3	3	7	.....	5	.....	.....	.....	40
Salmonella	.....	.....	.....	.....	5	.....	3	+	25
Staphylococcus	.....	.....	.....	.....	5	.....	3	+	.....
Corynebacterium	.....	.....	.....	.....	35	.....	.....	12	.....
Hemophilus	.....	.....	.....	.....	.....	.....	3	10	5
Diphtheroids	.....	.....	.....	.....	25	+	.....	.....	.....
Diplococci	.....	.....	6	.....	.....	.....	.....	.....	6
Coliforms	.....	.....	.....	.....	10	.....	.....	+	.....
Pseudomonas	.....	.....	.....	.....	3	.....	.....	.....	3
Listeria	.....	.....	.....	.....	.....	.....	.....	.....	1

* P = grossly pneumonic; N = grossly normal. + = Less frequently recovered organisms in unstated percentages.

amniotic sac, and from mice inoculated by the intranasal route. This virus was recovered from the lungs of swine 17 days postinoculation (PI) and from the trachea 52 days PI.

The initial studies on virus pneumonia of pigs (VPP) have been reviewed by Betts [6] and Hjarre.[15] Since these reviews, a number of reports have been published concerning the recovery of viral agents from this disease. Palyusik [21] recovered an agent from 11 of 82 pneumonic lungs. This agent produced pock lesions on the chorioallantoic membrane of chicken embryos. The inoculation of suspensions of infected chorioallantoic membranes into young pigs produced mild pneumonia.

Wesslen and Lannek [42] recovered a cytopathogenic agent, in primary swine lung cell cultures, from six of eight pneumonic swine lungs. The agent was susceptible to the tetracycline antibiotics but it was not neutralized by serum from naturally or experimentally infected swine. Experimental inoculation of swine with this agent produced only enlargement and edema of the pulmonary and mediastinal lymph glands. Histologic studies [17] of the cytopathic changes produced by this agent revealed minute intracellular and extracellular particles which did not resemble organisms of the psittacosis lymphogranuloma venereum group. Hjarre *et al.*[14] obtained only transient cytopathic changes from three of the 16 pneumonic lung specimens they inoculated into swine cell cultures. Lannek and Wesslen [19] inoculated pneumonic lung material into suspended cell cultures and found that eighth passage culture fluids produced gross and histologic lesions of pneumonia in swine. They recovered the cytopathogenic agent of swine enzootic pneumonia (SEP) from the experimentally produced pneumonic lesions. Intraperitoneal inoculation of swine with this agent produced peritonitis, pleuritis, and pericarditis. Dinter *et al.*[8] found yolk-sac inoculation of 5- to 7-day chicken embryos more effective than cell cultures in recovering the SEP agent. Fontaine *et al.*[11] repeated Dinter's results and succeeded in reproducing the disease in pigs with third passage yolk-sac suspensions. Fontaine *et al.*[11,12] reported that intraperitoneal inoculation of swine with pneumonic lung material irregularly produced an acute disease characterized by pericarditis, pleuritis, peritonitis, and arthritis.

Romvary and Csontos [24] recovered a virus from swine with subacute pneumonia by inoculation of 6- to 7-day chicken embryos. The agent was susceptible to the broad-spectrum antibiotics, did not agglutinate erythrocytes, and produced mononuclear infiltration in the lungs of experimentally infected pigs. In addition, they recovered an influenza-like virus from animals with acute pneumonia. Kojnok *et al.*[18] recovered a pseudorabies virus from the lungs, brains, and blood of swine and produced pneumonia in swine by intratracheal inoculation with the virus. Bakos *et al.*,[2] Englert,[10] and Terpstra *et al.*[36] have related inclusion body rhinitis, first described by Done,[7] to enzootic pneumonia of swine.

Two additional viral agents have been incriminated as causes of pneumonia in swine. Verlinde and Versteeg [41] recovered a type A5 Coxsackie virus from swine in a localized outbreak of pneumonia involving both children and swine. Convalescent serum from both the children and the swine neutralized the virus. Sibalin and Lannek [30] recovered a cytopathogenic swine enteric virus in pig kidney cell cultures. Inoculation of this virus into colostrum-deprived pigs produced a nonpurulent pneumonitis and encephalomyelitis.

Materials and Methods

Swine lungs were collected from various packing plants in Iowa, from the Iowa Veterinary Diagnostic Laboratory and, in a few cases, from experimentally infected animals. These specimens were usually refrigerated only a few hours prior to being cultured for bacteria and *Mycoplasma sp.* Portions were fixed in Bouin's solution for histopathologic examination, and the remainder was frozen at −20 C. The inoculum was prepared either by mincing with scissors or by grinding in a glass tissue grinder with Dulbecco's [8] phosphate buffer at an approximate 1:10 dilution. The preparations were then centrifuged at 2,000 r.p.m. for 20 minutes, and the supernatant fluids were either filtered through cellulose membranes* of 0.65 μ average pore diameter (APD), or treated with 1,000 units of penicillin and 1,000 μg. of streptomycin per milliliter for one hour prior to inoculation into cell cultures, chicken embryos, mice, or swine.

Bacteriologic studies were carried out by streaking pieces of lung tissue or a loopful of expressed fluids over 5% horse-blood agar plates. The inoculated plates were then streaked with a *Micrococcus sp.*, previously shown to enhance the growth of *Hemophilus influenzae suis.* The plates were incubated for 24 to 48 hours at 37 C. Bacteria were identified by routine morphologic and biochemical methods. *Mycoplasma hyorhinis* was isolated in beef-heart infusion turkey-serum medium [34] inoculated with tissue or expressed fluids from the lung specimens. Identification of these organisms was carried out according to the methods of Switzer.[34]

Primary swine-kidney, and primary swine-lung cell cultures were prepared according to the method of Switzer.[39] All specimens were passaged in a second series of cell cultures before being considered negative for the presence of cytopathogenic agents.

Chicken embryos 8 to 10 days old were inoculated with lung suspension by either the allantoic or amniotic sac routes and, in all cases, by the yolk-sac route. All specimens were passaged a second time before being considered negative.

Carworth Farm (CWF) mice, 3 to 4 weeks old, were inoculated with pneumonic lung suspensions by both the intranasal and intraperitoneal routes while under light ether anesthesia. The mice were observed for ten to 14 days, after which they were killed with chloroform and necropsied. Lung and spleen tissues were collected from all groups of mice and suspended at a 1:10 dilution in Dulbecco's phosphate buffer solution. One additional passage was made in 4 more mice before the specimen was considered negative.

Swine used in the experiments were from a disease-controlled swine herd, maintained in quarantine. This herd has been free of detectable respiratory disease since its establishment in 1955.

In order to establish that the systems used would detect swine influenza virus, 2 ml. of Shope's Iowa 15 strain of swine influenza virus was instilled intranasally in 4 pigs 3 days old which were then kept in isolation. Two animals were killed five days PI. These pigs had small areas of light, plum-colored consolidation involving the right anterior and cardiac lobes of the lung. The 2 remaining animals, killed 16 days PI, had normal lungs. The lungs of these 4 animals were negative for *M. hyorhinis* and bacteria. Portions were ground and filtered through a cellulose membrane of 0.65 μ APD and inoculated into swine-kidney cell cultures, chicken embryos, and mice. Swine influenza virus was recovered from the lungs of the 2 pigs killed five days PI. The virus was recovered in cell cultures, chicken embryos inoculated by the chorioallantoic route, and in mice inoculated intranasally. The 2 pigs examined 17 days PI failed to yield swine influenza virus.

Three 8-week-old pigs were inoculated intratracheally with 2 ml. of a 0.65 μ APD cellulose-membrane filtrate of pneumonic lung suspension from the fourth passage of VPP strain of VMRI No. 1. Three control pigs were inoculated with 2 ml. of unfiltered pneumonic lung suspension. The animals were killed 21 days PI. Two of the 3 virus-control pigs had small pneumonic foci scattered throughout the anterior and cardiac lobes. One of the 3 filtrate-inoculated pigs had an early diffuse consolidation throughout the lungs. No gross lesions were found in 1 virus control and 2 filtrate-inoculated pigs. Histopathologic examination revealed changes typical of VPP in the grossly pneumonic lungs.

Results

After establishing that the systems used were capable of detecting swine influenza virus in pneumonic swine lungs and that the cellulose membrane filters allowed passage of the agent of VPP, a survey of the viruses, bacteria, rickettsias, and *Mycoplasma sp.* present in 86 pneumonic and 15 grossly normal swine lungs, was undertaken.

Only one viral agent (L5) was recovered from the 86 pneumonic specimens. No virus was recovered from the 15 grossly normal lungs. Virus L5 was recovered in swine-kidney cell cultures where it pro-

* Millipore Filter Corporation, Bedford, Mass.

duced a severe CPE on initial passage. The CPE produced by this agent was identical to the type 3 described by Switzer *et al.*[37] This agent was not pathogenic for chicken embryos or mice. The intratracheal inoculation of fluids from the second swine-kidney cell-culture passage of agent L5 failed to produce either symptoms or lesions in 4 pigs. Nasal and rectal swabs collected at necropsy four weeks PI failed to yield the virus when inoculated into swine-kidney cell cultures.

Mycoplasma hyorhinis was recovered from 44 of the 86 pneumonic lungs, but from only one of the 15 grossly normal lungs. Pure cultures of *M. hyorhinis* and *Past. multocida* were recovered in combination from 21 specimens. In 8 of these 21 lungs, other organisms were present. *Streptococcus sp.* were recovered in combination with *M. hyorhinis* in 11 specimens. Although most of the *M. hyorhinis* isolates were recovered in beef-heart infusion turkey-serum medium, several isolates were recovered only in cell cultures or chicken embryos inoculated by the yolk-sac route. These isolates could subsequently be grown in beef-heart infusion turkey-serum medium. They produced a CPE in swine-kidney cell cultures which was similar to the type 1 of Switzer *et al.*[37] Frequently, the CPE was not observed until one additional passage had been made and, in one case, no effect was seen until the fourth consecutive 7-day passage in swine-kidney cell cultures. The mortality pattern and lesions produced in chicken embryos resembled those described by Switzer.[34]

Pasteurella multocida was recovered from 35 of 86 pneumonic lungs. No isolates were recovered from the normal lungs. Seven of the *Past. multocida* isolates were in pure culture, while 21 were recovered in conjunction with *M. hyorhinis*, and nine in conjunction with *Streptococcus sp.*

The streptococci recovered were grouped according to the type of hemolysis produced on horse-blood agar. Fifteen *beta*-hemolytic streptococci were recovered from the pneumonic lungs and none were recovered from the normal lungs. None of the *beta*-hemolytic streptococci were recovered in pure culture. Mixed infections occurred with *M. hyorhinis* in 7 animals, and with staphylococci in 9. Fifteen *alpha*-hemolytic streptococci were recovered from the 86 pneumonic lungs. These streptococci appeared to be similar in morphology, growth characteristics, and type of hemolysis, but biochemical studies revealed the group to be markedly heterogeneous. Five strains of the *alpha*-hemolytic streptococci were recovered from the normal lung specimens and proved equally difficult to classify. Six of the 15 isolates from the pneumonic lungs were recovered in conjunction with *M. hyorhinis*, four in conjunction with *Past. multocida*, and seven in conjunction with staphylococci. Five nonhemolytic streptococci were recovered from the pneumonic lungs. Two of these were recovered in pure culture while the three other isolates were recovered in conjunction with a number of other organisms.

Corynebacterium pyogenes was recovered from three pneumonic specimens but none was in pure culture. In one specimen from which *Past. multocida* and *C. pyogenes* were recovered, the lung, in addition to showing extensive areas of plum-colored consolidation, was covered with hobnail abscesses varying from 1 to 3 cm. in diameter.

Organisms closely resembling *Corynebacterium renale* were recovered from three pneumonic specimens. None of these were present in the lung in pure culture.

Other organisms recovered were *Corynebacterium sp., H. influenzae suis, Bordetella bronchiseptica, Salmonella typhimurium, Escherichia coli, Proteus sp., Listeria monocytogenes,* and *Staphylococcus sp.* None of these organisms were recovered in pure culture, and in most cases they appeared as only a few colonies on the original cultures. The organisms recovered are listed in Table 2.

TABLE 2—Organisms Recovered from 86 Pneumonic and 15 Grossly Normal Swine Lungs

Organisms recovered	86 Pneumonic lungs		15 Grossly normal lungs	
	No.	%	No.	%
Negative for organisms	10	11.6	7	46.6
Rickettsia	0	0	0	0
Virus	1	1.1	0	0
Mycoplasma hyorhinis	44	51.1	1	6.6
Pasteurella multocida	35	40.7	0	0
Streptococcus spp.	35	40.7	5	33.3
Corynebacterium pyogenes	3	3.5	0	0
Corynebacterium renale	3	3.5	0	0
Corynebacterium-like	1	1.1	3	20.0
Hemophilus influenzae suis	2	2.3	0	0
Bordetella bronchiseptica	1	1.1	2	13.3
Salmonella typhimurium	1	1.1	0	0
Escherichia coli	8	9.3	3	20.0
Proteus sp.	4	4.6	2	13.3
Listeria monocytogenes	1	1.1	0	0
Staphylococcus spp.	20	23.2	8	53.3

Discussion

Viruses capable of producing pneumonia in swine were not recovered from the lung specimens, by means of the detection systems used. In all probability, a high percentage of the pneumonic specimens harbored the agent of VPP, but no attempt was made to ascertain its presence by inoculation of susceptible swine. Recovery of swine influenza virus was not made from any specimen, even though some of the specimens were collected during the season when the disease is said to be prevalent. However, since swine influenza virus is present in the pneumonic lesions for only the first few days, some of the lesions examined could have been initiated by this agent. Nonetheless, it seems unlikely that all of the collections would have been made at this stage. In only one case was a detectable virus (L5) recovered in swine cell cultures. No detectable viral or rickettsial agents were recovered in chicken embryos or mice. The viral agent (L5) may have been a contaminant from either the nasal or the oral cavity. This virus was not pathogenic for swine. These findings agree with the conclusion of Hjarre *et al.*[14] that viral cytopathogenic agents are not easily recovered from animals infected with swine pneumonia.

The high incidence of *M. hyorhinis* indicates that the effects of this agent as a secondary invader in swine pneumonia should be determined. This organism regularly passed filters of minimum pore size for the passage of the agent of VPP, and it is not affected by high levels of penicillin and streptomycin. While this organism is not of primary etiologic importance, it poses a serious problem in studies on swine pneumonia since at the present time it is impossible to rid experimental inoculums of this organism without also eliminating the virus. The cytopathic changes produced by *M. hyorhinis* closely resemble those changes described by Lannek and Wesslen.[17] These authors [18] considered the possibility that the cytopathogenic SEP agent they recovered might be a *Mycoplasma*.

The bacteriologic findings in this study parallel and supplement the results obtained in similar surveys by other authors (Table 1). Only 12% of the pneumonic but 47% of the normal specimens were bacteriologically negative. *Pasteurella multocida* and *Streptococcus sp.* were the predominant bacteria recovered from the pneumonic lungs, while *Past. multocida* was not cultured from the grossly normal lungs. The only organisms recovered in apparently significant numbers from the normal lungs were *alpha*-hemolytic streptococci.

References

[1] Bakos, K.: The Cytopathogenicity of Two Different Strains of Swine Influenza Virus. Nord. Vet.-med., 9, (1957): 369–372.

[2] Bakos, K., Obel, A-L., Swahn, O., and Walzl, H.: "Inclusion-Body-Rhinitis" bei Ferkeln mit experimenteller enzootischer Pneumonie. Zentralbl. Vet.-med., 7, (1960): 262–272.

[3] Betts, A. O.: Pneumonic Diseases of the Pig Caused by Viruses. A Review of Literature. Brit. Vet. J., 109, (1953): 99–106.

[4] Carter, G. R., and Schroder, J. D.: Pleuropneumonia-like Organisms Associated with Pneumonia in Swine. Canad. J. Comp. Med., 19, (1955): 219–220.

[5] Carter, G. R., and Schroder, J. D.: Virus Pneumonia of Pigs in Canada, with Special Reference to the Role of Pleuropneumonia-like Organisms. Cornell Vet., 46, (1956): 344–354.

[6] Dinter, Z., Wesslen, T., and Lannek, N.: Die Kultivierung des SEP-Agens im Dottersack des Bruteies. Zentralbl. Vet.-med., 4, (1957): 581–587.

[7] Done, J. T.: "Inclusion Body" Rhinitis of Pigs. Vet. Rec., 67, (1955): 525–528.

[8] Dulbecco, R., and Vogt, M.: Plaque Formation and Isolation of Pure Lines of Poliomyelitis Virus. J. Exptl. Med., 99, (1954): 167–182.

[9] Ellis, E. M., and Schroeder, W. F.: *Chromobacterium violaceum* Infection in Swine. Vet. Med., 53, (1958): 578–579.

[10] Englert, H. K.: Done'sche Einschlusskörperchen beim Schwein, enzootische Pneumonie der Schweine und Schnüffelkrankheit. Deutsche tierärztl. Wchnschr., 67, (1960): 401–402.

[11] Fontaine, M., Fontaine, Mme. M., Goret, P., Brion, A., and Pilet, C.: Une enzootie de pneumonie à virus du porc dans la région parisienne. Isolement et essai d'identification du virus en cause. Bull. Acad. vét. France, 31, (1958): 451–471.

[12] Fontaine, M., Goret, P., Brion, A., Pilet, C., Girard, M., and Alloui, P.: Nouvelles recherches expérimentales sur la pneumonie à virus du porc. Bull. Acad. vét. France, 32, (1959): 675–694.

[13] Forray, A.: A PPLO-csoportha tartozo csirak elofordulasa sertesekben. Magyar allator. Lap., 14, (1959): 329–331.

[14] Hjärre, A., Dinter, Z., and Bakos, K.: Über Züchtungsversuche mit dem Schweineinfluenzavirus (Shope) und dem Virus der enzootischen Schweinepneumonie in vitro. Nord. Vet.-med., 6, (1954): 919–935.

[15] Hjärre, A.: Über die enzootische Viruspneumonie beim Schwein. Monatsh. Vet.-med., 12, (1957): 503–508.

[16] Kojnok, J., Olah, P., and Surjan, J.: Adatok sertesek endemias tudogyulladasanok koroktanakoz. Magyar allator. Lap., 14, (1959): 262–266.

[17] Lannek, N., and Wesslen, T.: Histological Examinations of Tissue Cultures Inoculated with a Cytopathogenic Agent from Swine Enzootic Pneumonia. Acta path. et microbiol. Scand., 34, (1955): 343–351.

[18] Lannek, N., and Wesslen, T.: Evidence that the SEP Agent is an Etiological Factor in Enzootic Pneumonia of Swine. Nord. Vet.-med., 9, (1957): 177–190.

[19] Matthews, P. R. J., and Pattison, I. H.: The Identification of a Hemophilus-like Organism Associated with Pneumonia and Pleurisy in the Pig. J. Comp. Path. & Therap., 71, (1961): 44–52.

[20] Morcos, Z., Zaki, O. A., and Zaki, R.: Bacteriological Study of Swine Pneumonia in Egypt. Vet. Med., 42, (1947): 255–258.

[21] Palyusik, M.: Untersuchungen über die Ätiologie der enzootischen Ferkelpneumonie. Acta vet. Hungary, 3, (1953): 63–73.

[22] Pattison, I. H., Howell, D. G., and Elliot, J.: A Haemophilus-like Organism Isolated from Pig Lung and the Associated Pneumonic Lesions. J. Comp. Path. & Therap., 67, (1957): 320–330.

[23] Rislakki, V.: Om den i Finland förekommande smittosamma grishostans etiologi. I. Infektionsförsök met forsöksgrisar. Nord. Vet.-med., 5, (1953): 113–124.

[24] Romváry, J., and Csontos, V.: Adatok a sertés vírusos eredetü hurutos, hurutosgennyes tüdögyulladásához. Magyar allator. Lap., 15, (1960): 210–214.

[25] Ryu, E.: A Bacteriological Study of Swine Infectious Pneumonia. General Bacteriological Examination and Experiments on Artificial Infection. Coll. of Agric., National Taiwan Univ. Memoirs, 3, (1954): 1–20.

[26] Sasahara, J., Hayashi, S., Kumagai, T., Yamamoto, Y., Hirasawa, N., Munekata, K., Okaniwa, A., and Kato, K.: On a Swine Virus Disease Newly Discovered in Japan. Its Characteristic Traits of Pneumonia. I. Isolation of the Virus. II. Some Properties of the Virus. Jap. J. Vet. Res., 4, (1954): 131–139.

[27] Sasahara, J., Hayaski, S., Kumagi, T., Hirasawa, K., Munekata, K., Okaniwa, A., and Kato, K.: A Swine Disease Newly Discovered in Japan. Its Characteristic Traits of Pneumonia. III. The Experimental Infection for Young Swine. Jap. J. Vet. Res., 4, (1954): 297–301.

[28] Sasahara, J., Kumagai, T., and Kato, K.: A Swine Virus Disease Newly Discovered in Japan. Its Characteristic Traits of Pneumonia. IV. The Experimental Infection for Several Laboratory Animals. Jap. J. Vet. Res., 4, (1954): 302–308.

[29] Shope, R. E.: Swine Influenza (Flu, Hog Flu, Swine Flu). Diseases of Swine. Iowa State University Press, Ames, (1958): 81–98.

[30] Sibalin, M., and Lannek, N.: An Enteric Porcine Virus Producing Encephalomyelitis and Pneumonitis in Baby Pigs. Arch. Virusforsch., 10, (1960): 31–45.

[31] Sippel, W. L., Medina, G., and Atwood, M. B. Outbreaks of Disease in Animals Associated with *Chromobacterium violaceum*. I. The Disease in Swine. J.A.V.M.A., 124, (June, 1954): 467–471.

[32] Spray, R. S.: The Bacteria in Normal and Diseased Lungs of Swine. J. Infect. Dis., 31, (1922): 10–21.

[33] Switzer, W. P.: A Suspected PPLO in Iowa Swine. Iowa Vet., 25, (1954): 9–11.

[34] Switzer, W. P.: Studies on Infectious Atrophic Rhinitis. IV. Characterization of a Pleuropneumonia-like Organism Isolated from the Nasal Cavities of Swine. Am. J. Vet. Res., 16, (Oct., 1955): 540–544.

[35] Switzer, W. P., Veterinary Medical Research Institute, Iowa State University, Ames, Iowa: Unpublished data, 1953–1958.

[36] Switzer, W. P.: Action of Certain Viruses, *Mycoplasma hyorhinis*, and Nasal Trichomonads on Swine Tissue Cultures. Am. J. Vet. Res., 20, (Nov., 1959): 1010–1019.

[37] Switzer, W. P., and L'Ecuyer, C.: Detection of Swine Nasal Viruses in Cell Cultures. Am. J. Vet. Res., 21, (Nov., 1960): 967–970.

[38] Terpstra, J. E., and Akkermans, J. P. W. M.: Enkele acentekiningen over atrofische rinitis. Tijdschr. Diergeneesk, 85, (1960): 1222–1233.

[39] Thal, E., and Rutqvist, L.: The Pathogenicity of *Corynebacterium equi* for Pigs and Small Laboratory Animals. Nord. Vet.-med., 11, (1959): 298–304.

[40] Thorp, F., Jr., and Tanner, F. W.: A Bacteriological Study of the Aerobic Flora Occurring in Pneumonic Lungs of Swine. J.A.V.M.A., 96, (Feb., 1940): 149–159.

[41] Verlinde, J. D., and Versteeg, J.: Coxsackie-viruspneumonie bij biggen als smetstofbron voor de mens. Tijdschr. Diergeneesk., 83, (1958): 459–468.

[42] Wesslen, T., and Lannek, N.: The Isolation and Cultivation in Tissue Culture of a Cytopathogenic Agent from Pigs with Enzootic Pneumonia (So-called Virus Pneumonia). Nord. Vet.-med., 6, (1954): 481–499.

[43] Young, G. A., and Underdahl, N. R.: An Evaluation of Influenza in Midwestern Swine. Am. J. Vet. Res., 16, (Oct., 1955): 545–552.

[44] Young, G. A.: Virus Pneumonia of Pig (VPP). Diseases of Swine. Iowa State University Press, Ames (1958): 99–106.

SUMMARIO IN INTERLINGUA

Un Studio Microbiologic de Pneumonic e Normal Pulmones Porcin

Es presentate le resultatos del examine de 86 pneumonic e 15 normal pulmones porcin pro virus, bacterios, rickettsias, e *Mycoplasma sp.* Nulle agente virusal esseva trovate in le pulmones normal e solmente un in le pulmones pneumonic. Illo esseva cytopathogenic in culturas de cellulas de ren porcin, sed in experimentos con porcos illo produceva nulle lesiones. *Mycoplasma hyorhinis* esseva trovate in 51,1% del pulmones pneumonic e in 6,6% del pulmones normal. *Pasteurella multocida* e *Streptococcus sp.* esseva le major bacterios trovate in le pulmones pneumonic, durante que solmente streptococcos hemolytic alpha appareva in numeros significative in le pulmones normal. Nulle rickettsias esseva trovate. Nulle virus de influenza porcin esseva isolate.

Serum Transaminase and Aldolase During Migration of Larval *Ascaris suum* in Swine

M. F. Andrews, B.S., D.V.M.; P. K. McIlwain, B.S.; D. F. Eveleth, Ph.D., D.V.M.

SUMMARY

Serum glutamic-oxaloacetic transaminase (SGO-T), serum glutamic-pyruvic transaminase (SGP-T), and serum aldolase levels in swine infected experimentally with *Ascaris suum* were studied. The SGP-T level rose distinctly on the first day of infection and reached a peak concentration at about three days. The concentration was elevated for about two weeks.

The glutamic-oxaloacetic transaminase and the aldolase levels also had an early increase, but had a second peak of concentration near or during the second week after infection.

INTRODUCTION

During an investigation of *Ascaris suum* infections in swine, an attempt was made to determine whether larval migrations were taking place in the host tissues. The effect of migrating ascarid larvae on transaminase and aldolase levels in the serum of swine was studied.

Review of Literature

Clinical interest in the transaminases has been centered mainly on the serum glutamic-oxaloacetic transaminase (SGO-T) and the serum glutamic-pyruvic transaminase (SGP-T). Accurate colorimetric methods for the determination of these enzymes have been devised.[1, 7] In man, these transaminases have proved valuable in diagnosing and studying cardiac and liver diseases. In heart disease, the SGO-T level is elevated when there is necrosis of the myocardium, but the SGP-T level is elevated only slightly, if at all. When there is destruction of the hepatic tissue in liver disease, both the SGO-T and the SGP-T levels are markedly elevated. In a review of the transaminases, Schloss[6] discusses these and other abnormal conditions that elevate the serum transaminase level.

Received for publication Aug. 17, 1960.

From the Agricultural Experiment Station and Department of Veterinary Science, North Dakota Agricultural College, Fargo.

Cornelius *et al.*[2] attempted to determine normal levels of SGO-T and SGP-T in the serums of healthy horses, cattle, swine, dogs, and chickens. They also studied the levels of the GO-T and GP-T in selected tissues from the same animals, and the SGO-T and SGP-T levels in the 4 mammals after administration of carbon tetrachloride. In only the dog was there a marked increase in SGP-T activity when the liver was damaged with carbon tetrachloride. Other workers have also studied the levels of the transaminases in the serum, plasma, and tissues of pigs in diseased[4] and normal[6] conditions.

The aldolase content of serum has also been used to study diseases in which cellular necrosis occurs. Harris[3] published a table summarizing some of the work that has been done with this enzyme. She also compared five methods of determining aldolase content of the serum and discussed the method that yielded the best results.

Materials and Methods

Reagents sold as a kit* were used in the transaminase determinations. These reagents are based on the methods of Cabaud *et al.*[1] and Wroblewski and Cabaud.[7] The test for SGO-T uses a solution of aspartic and *alpha*-ketoglutaric acids buffered to pH 7.5 as a substrate and utilizes dinitrophenylhydrazine as a color developer. The test for SGP-T is similar, but utilizes a substrate of alanine and *alpha*-ketoglutaric acid buffered to pH 7.5.

In these determinations, the enzymes are calculated in units per milliliter of serum. The test for SGO-T depends on the formation of oxaloacetic acid from aspartic acid. The oxaloacetic acid breaks down to form pyruvic acid and the pyruvic acid unites with the dinitrophenylhydrazine to form a chromogenic material. The SGP-T converts alanine to pyruvic acid. Under the conditions of the test, 1 unit of SGO-T or SGP-T represents the formation of chromogenic material equivalent to 1 μg. of pyruvate.

Serum aldolase was determined by the method of Harris.[3] The serum sample is incubated with fructose-1,6-diphosphate and hydrazine to form triose phosphates. The color reagent is 2,4-dinitrophenylhydrazine, which forms a color derivative of the triose phosphates that can be read at 540 mμ on a spectrophotometer. The aldolase is measured in units that equal the amount of enzyme that will split 1.518 μg. of fructose-1,6-diphosphate per milliliter of serum per hour at 38 C.

* Dade Reagents, Inc., Miami, Fla.

Ascarid ova were harvested from mature ascarides collected from swine at a local slaughterhouse. The ova were embryonated by bubbling air through a solution of 2% potassium dichromate into which the ova had been placed. When embryonation was sufficient, as demonstrated by microscopic examination, the ova were diluted with water and counted.

The experiment was conducted in two trials, each utilizing 10 swine of both sexes. Each swine weighed 35 to 45 lb. at the start of the trials. In both trials, the animals were placed at random into two lots of 5 swine each. Those in one lot served as noninfected controls. The swine in the other lot were each given approximately 500 embryonated ova of *A. suum* in gelatin capsules. Blood samples were taken at regular intervals from the anterior vena cava. The serum was collected and the transaminase and aldolase contents were determined.

Results

The results of the trials are illustrated graphically in Figures 1, 2, and 3.

The SGP-T levels are shown in Figure 1. This enzyme reached a peak concentration in the serum of infected swine 30 to 60 hours after the ova were administered. This early peak was the only definite peak demonstrated with SGP-T.

The SGO-T levels are shown in Figure 2. A peak in concentration in the infected swine occurred within 30 to 90 hours, but it was followed by a second peak 210 to 250 hours after the ova were administered.

The serum aldolase levels are shown in Figure 3. The increase in the aldolase level during migration of the larvae also is evident, and a double peak in the enzyme concentration is shown. The second peak appeared earlier than the second peak of SGO-T concentration.

Discussion

A definite increase in SGP-T, SGO-T, and aldolase levels during the migration of *A. suum* was demonstrated. Cornelius [2] found that the SGP-T activity was not significantly increased in swine after carbon tetrachloride poisoning and found a low level of GP-T in the liver tissue. Orstadius [4] found that there was a definite increase in SGP-T

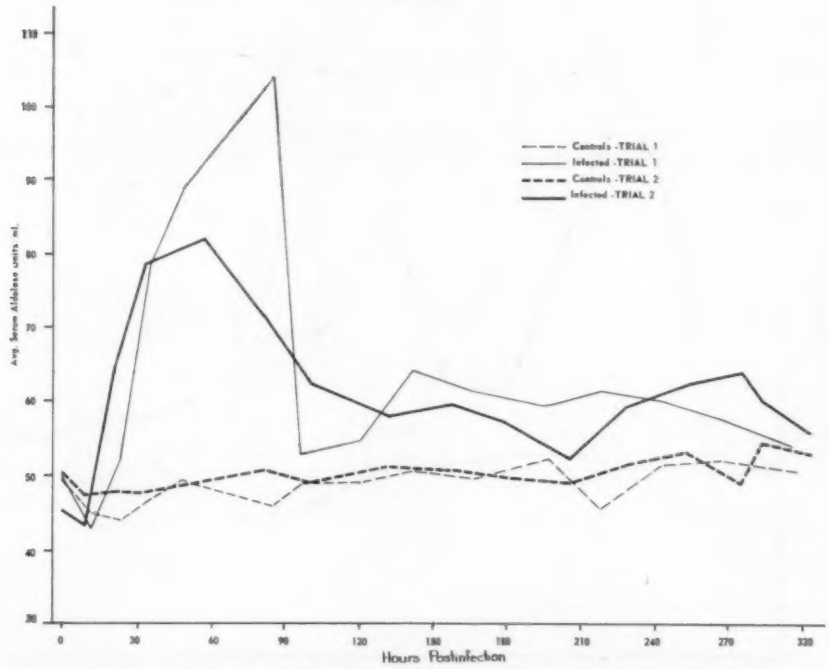


Fig. 1—Serum glutamic-pyruvic transaminase levels in swine during acute Ascaris suum infection.

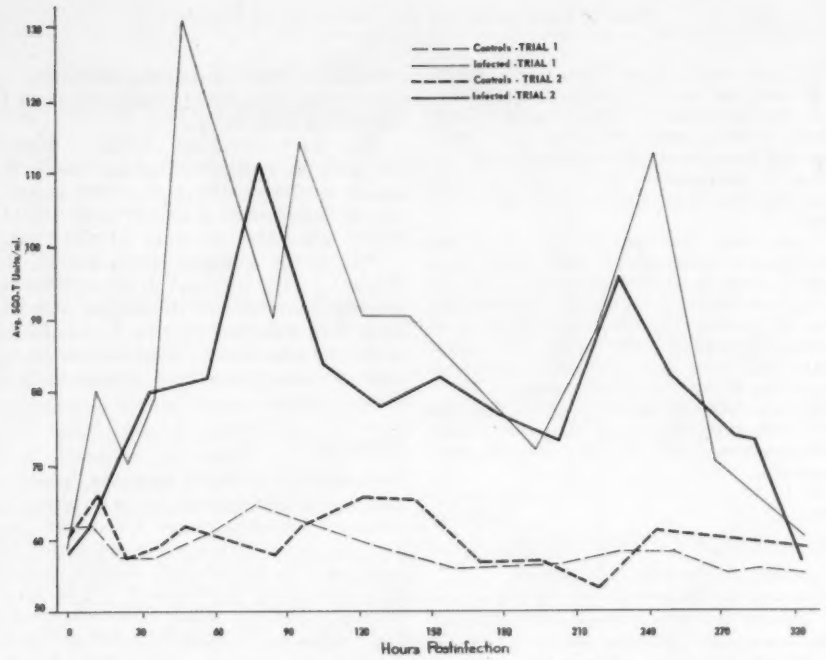


Fig. 2—Serum glutamic-oxaloacetic transaminase levels in swine during acute Ascaris suum infection.

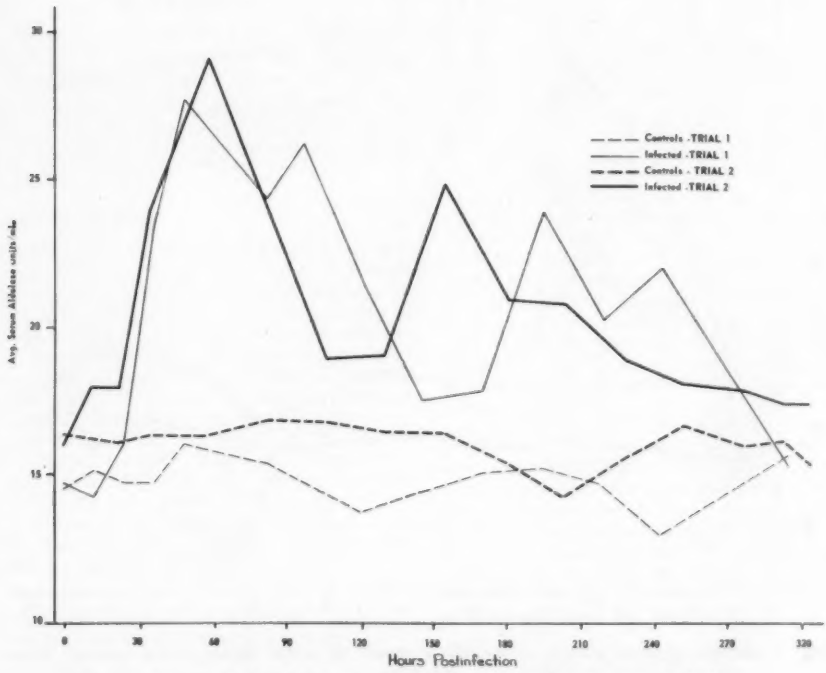


Fig. 3—Serum aldolase levels in swine during acute Ascaris suum infection.

following liver dystrophy in swine, and Wretlind [6] found that the livers of swine contained a moderate amount of GP-T. The results of these studies indicate a definite rise in SGP-T during the migration of the ascarid larvae within the liver.

The second rise in the SGO-T and serum aldolase seems to occur during the establishment of larval infection in the lungs, although the second rise in aldolase occurs earlier than that in SGO-T.

These enzymes may possibly be used to demonstrate the migration of *A. suum* in swine.

References

[1] Cabaud, P., Leeper, R., and Wroblewski, F.: Colorimetric Measurement of Serum Glutamic Oxaloacetic Transaminase. Am. J. Clin. Path., 26, (Sept., 1956): 1101–1105.

[2] Cornelius, C. E., Bishop, J., Switzer, J., and Rhode, E. A.: Serum and Tissue Transaminase Activities in Domestic Animals. Cornell Vet., 49, (Jan., 1959): 116–126.

[3] Harris, Sister Mary Laureen: Serum Aldolase Determination as a Routine Laboratory Procedure. Am. J. Med. Technol., 24, (March, 1958): 99–105.

[4] Orstadius, K., Wretlind, B., Lindberg, P., Nordstrom, G., and Lannek, N.: Plasma Transaminase and Transferase Activities in Pigs Affected with Muscular and Liver Dystrophy. Zentralbl. Vet.-med., 6, (1959): 971–980.

[5] Schloss, M. F.: A Résumé on Transaminase. Am. J. Med. Technol., 25, (Jan., 1959): 47–52.

[6] Wretlind, B., Orstadius, K., and Lindberg, P.: Transaminase and Transferase Activities in Blood Plasma and in Tissues of Normal Pigs. Zentralbl. Vet.-med., 6, (1959): 963–970.

[7] Wroblewski, F., and Cabaud, P.: Colorimetric Measurement of Serum Glutamic Pyruvic Transaminase. Am. J. Clin. Path., 27, (Feb., 1957): 235–239.

SUMMARIO IN INTERLINGUA

Transaminase e Aldolase del Sero in Porcos Durante le Migration de Larvas de *Ascaris suum*

Esseva studiate le nivellos seral de transaminase glutamic-oxaloacetic (T-GOS), de transaminase glutamic-pyruvic (T-GPS), e de aldolase in porcos experimentalmente inficite con *Ascaris suum*. Le nivello de T-GPS montava nettemente a partir del prime die post le infection e attingeva su maximo post circa tres dies. Le concentration de T-GPS remaneva elevate durante circa duo septimanas.

Le nivellos de T-GOS e de aldolase etiam monstrava un prompte augmento, sed illos habeva un secunde culmine de concentration circum o durante le secunde septimana post le infection.

Studies on Meningiomas in Cats

Hansruedi Luginbuhl, Dr. med. vet.

SUMMARY

One or more meningiomas were found in eight of 155 cat brains carefully examined at necropsy. In 7 cats, 8 to 17 years old, the tumors were in the tela choroidea of the third ventricle and were histologically diverse in character. Endotheliomatous, psammomatous, fibroblastic, and mixed endotheliomatous-fibroblastic variants were diagnosed.

In two of the brains, there were meningeal proliferations on the corpus callosum in addition to the meningiomas which extended into the third ventricle; in two other cases there were numerous small nodules on the convexity of the cerebral hemispheres.

Attention is drawn to the value of systematic examination of the brains of all animals examined postmortem.

INTRODUCTION

Detailed examination of the brains of animals at necropsy, even in the absence of clinically detectable nervous signs, is of considerable value from the standpoint of comparative neuropathology. Only after thorough examination and testing of a large amount of material can one delineate the incidence and behavior of brain tumors in domestic animals. Although the occurrence of tumors in the brains of dogs and some other species is recorded in the literature,[1,6] the disproportionate frequency of feline meningioma has not been clearly recognized.

Materials and Methods

Between August, 1957, and July, 1958, complete necropsies with histologic studies were performed on 155 domestic cats. The brains were fixed in 10% formalin and the frontal planes were sectioned at 3 to 4 mm. In eight brains, small nodules were found, always in anatomical connection with the meninges or the choroid plexus of the third (and in one case, the fourth) ventricle. These growths were dumbbell-, forked-, or bell-shaped tumors, and varied in size from a maximum dimension of 12 mm. to nodules which were barely perceptible grossly or were of microscopic size and were almost indistinguishable in color from brain tissue. The topographic location of the tumors and their relationships with their surroundings will be described in detail under the individual cases. All of the tumors found in this study were meningiomas.

Review of Literature

A search through the collection at the Angell Memorial Animal Hospital and in the literature revealed three feline meningiomas large enough to have resulted in clinically detectable signs. One was located in the third ventricle and in the median fissure, and some impressive illustrations of it are depicted in Smith and Jones' text.[5]

The other two cases are described and illustrated in McGrath's text.[3] A 9-year-old male cat that circled and paced was found to have a large meningioma over the left cerebral hemisphere. In a 10-year-old female cat, clonic convulsive seizures were observed and examination of the brain revealed a large meningioma in relation to the third ventricle.

In his study on the cells of the arachnoid, Weed[9] described nodular masses involving the cerebral arachnoid in 2 cats. Upon opening the dura of the first animal, an old, weak cat, he found an exceptionally large "arachnoid cell cluster" that had all the characteristics of the so-called dural endothelioma of man. In the second cat, two obvious masses of new growth were apparent over the left sylvian fissure and attached to the falx cerebri. Both masses were histologically similar to the dural endothelioma of the first cat. The many features of similarity suggested to him that these true neoplasms were the

Received for publication Oct. 13, 1960.

From the Department of Comparative Neurology and Veterinary Ambulatory Clinic, University of Bern, Bern, Switzerland.

This study was supported by Grant B-1916 of the National Institute of Neurological Diseases and Blindness, U.S. Department of Health, Education and Welfare, Bethesda, Md.

The author was resident in pathology, under Dr. T. C. Jones, veterinary pathologist, from Aug. 1, 1957, to July 31, 1958, at the Angell Memorial Animal Hospital, Boston, Mass. The materials used in this study were collected at necropsies performed at the Angell Memorial Animal Hospital. Dr. Leon Z. Saunders translated the original manuscript into English. The histologic study reported herein was conducted under the guidance of Professors E. Frauchiger and W. Hofmann in the Department of Comparative Neurology of the Veterinary Ambulatory Clinic of the University of Bern.

proliferative end result of the arachnoid cell clusters that can be found in the arachnoid membrane of cats of advanced age.

Another meningioma in the cat was described by Cooper and Howarth.[1] In the course of some work on absorption of cerebrospinal fluid, they sectioned 60 cat brains serially and examined them histologically. They found pathologic alterations in the brains of 4 of these cats, none of which had had any clinical signs during the short observation periods of 12 to 36 hours. In addition to an astrocytoma, an oligodendroglioma and a granuloma, they found a "leptomeningioma" 0.2 mm. in diameter. The authors postulated that it was derived from a nest of arachnoideal cells, since it was similar in structure to a granulum meningicum.

Dahme[2] included one case in a cat in his publication on meningiomas in carnivores. He found a firm, pea-sized nodule on the cerebral dura mater in the right parietal region in a 10-year-old male cat. This tumor had caused atrophy of the underlying cerebral cortex. According to the classification of Henschen,[3] this would be a fibroblastic meningioma, giving the impression of a psammoma because of degenerative alterations, especially calcification. Monteagudo and Purpura[4] incidentally found a small meningioma compressing the cortex on both sides of the left posterior ectosylvian sulcus in a male cat of unknown age.

With so few recorded cases, it is pointless to attempt any discussion or conjectures regarding the incidence, significance, and types of meningiomas which occur in cats.

Reports of Cases

Nothing will be said here of the clinical illness which led to the death or euthanasia of the animals, since in no case was there any correlation with the meningiomas. As an aid to understanding the topography of the tumors, seemingly located in the third ventricle, their location is depicted diagrammatically in Figures 1 and 2.

Case 1.—A grayish-white, solid nodule was seen in the region of the superior colliculi of a 10-year-old spayed female Maltese (A 57-581). It extended under the corpus callosum and between the left and right Ammon's horns along the roof of the third ventricle, into which it projected (Fig. 3). The rostral end of the tumor was at the level* of the dorsal end of Ammon's horn. The conically shaped growth almost entirely filled the third ventricle, which was only slightly dilated. The dimensions in the fixed preparation were 6 by 3 by 3 mm. in the craniocaudal, lateral, and dorsoventral planes, respectively.

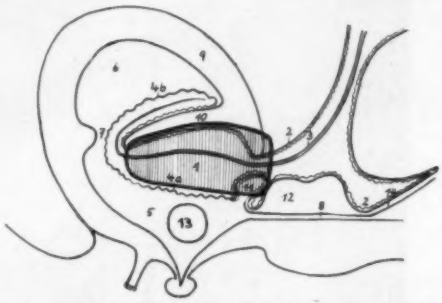


Fig. 1—Diagram of the topography of a meningioma projecting into the third ventricle of a cat (sagittal section).

The nodule was connected to the meninges by means of delicate strands of tissue extending from its dorsal third on both sides. There was a suggestion of a thin capsule around the tumor.

Histologically, the tumor had a clear pattern of lightly stained onionskin whorls, striae, and groups of meningothelial cells arranged in hook shapes as well as irregular strands of dark interstitial tissue arranged in rings and bands. The more lightly appearing cell whorls occurred primarily near the periphery. The tumor was strikingly avascular. Spear- and club-shaped calcium deposits were scattered in

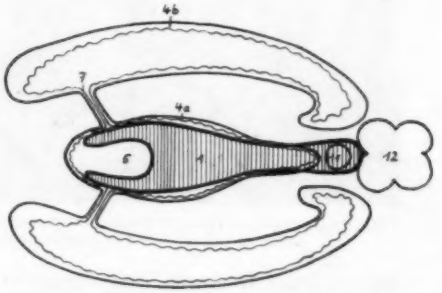


Fig 2—Diagram of the lateral and third ventricles, with the foramen of Monro and the plexus showing the extent of the meningioma: (1) tumor, (2) meninx, (3) vena magna cerebri, (4a) choroid plexus of third ventricle, (4b) choroid plexus of lateral ventricle, (5) third ventricle, (6) lateral ventricle, (7) foramen of Monro, (8) aqueduct of Sylvius, (9) corpus callosum, (10) fornix, (11) Pineal body, (12) colliculi, (13) massa intermedia, (14) anterior medullary velum.

* Throughout this report, "at the level" refers to sections in the frontal plane.

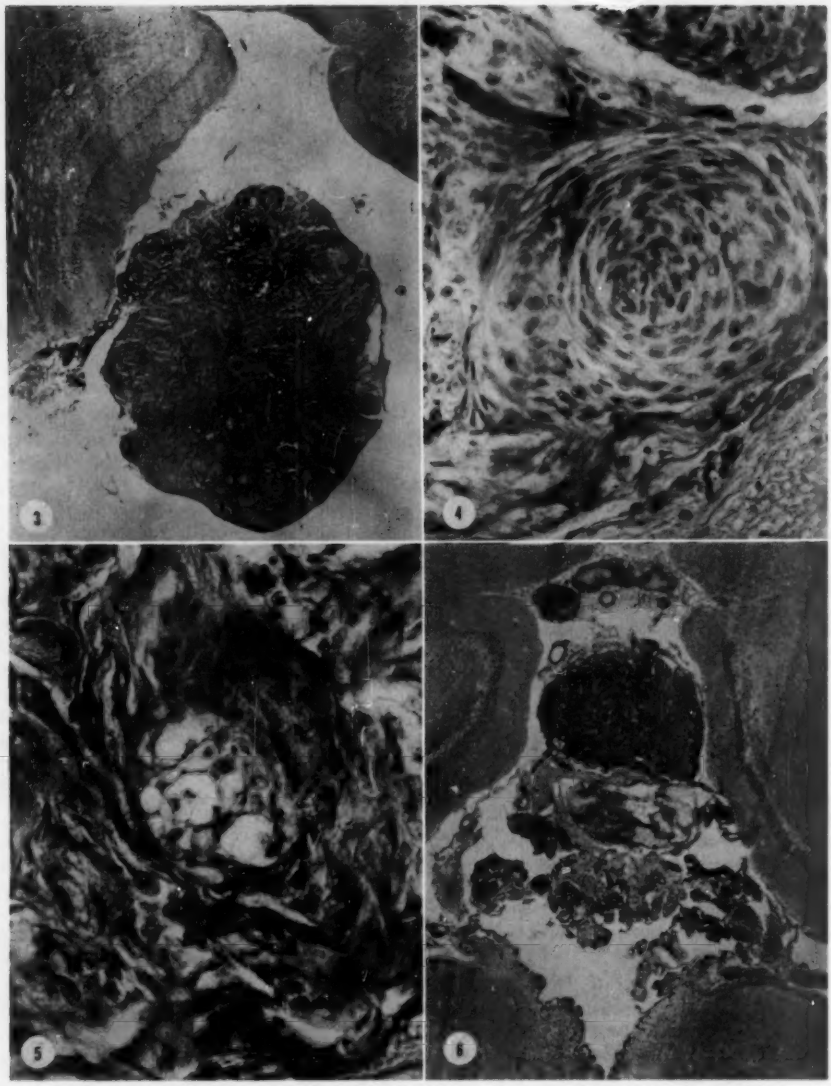


Fig. 3—Frontal section through meningioma located between the right and left Ammon's horns at the level of the anterior collicus. van Gieson stain; orig. mag. x 30.

Fig. 4—Whorl of cells at the periphery of a meningioma. "Onionskin" arrangement of meningothelial cells with chromatin-poor vesicular nuclei. There is a single needle-like calcium deposit in the surrounding stroma. H & E stain; orig. mag. x 320.

Fig. 5—Whorl with large vacuolated fat cells. van Gieson stain; orig. mag. x 450.

Fig. 6—Meningioma below the corpus callosum and connected with the meninx. The vena magna cerebri and choroid plexus are visible below the tumor. There are psammoma bodies on both sides of the plexus. H & E stain; orig. mag. x 35.

the interstitial tissue. The whorled structures contained 15 to 150 cells in cross section (Fig. 4). The cell borders were so indistinct that the cytoplasm of the cells appeared syncytially united. The scanty nuclear chromatin was often concentrated in a zone under the clearly visible nuclear membrane. Fine granules were present in many areas of the cytoplasm. The whorls were surrounded by several layers of collagen fibers, the nuclei of which appeared condensed and black; *i.e.*, pyknotic. The tumors generally contained a few capillaries, but no vessels were visible in the center of the whorls. Scattered through the whorls were concentric arrangements of cells resembling stromal cells. Some whorls contained shrunken and disintegrating cells with acidophilic cytoplasm and pyknotic nuclei. Hyaline degeneration in the center of the whorls was not noteworthy. Many whorls contained coarsely vacuolated fat cells or islets of fat cells (Fig. 5).

Apart from their whorled arrangement, the typical cells also occurred in smaller irregular aggregations and in strands. These were penetrated by stromal cells with pyknotic nuclei (Fig. 12).

Another nodule, about 1.5 mm. in diameter and barely visible macroscopically, was present in the median fissure at the level of the infundibulum. Its microscopic structure was similar to the tumor just described. Also noteworthy were some islets with coarsely vacuolated fat cells, in the neighborhood of which there were cell groups with small, chromatin-rich round to oval nuclei.

Both tumors were classified as endotheliomatous meningiomas with fibroblastic components.

Case 2.—A pear-shaped growth was grossly visible in the region of the epiphysis in a 16-year-old male cat (A 57-589). It tapered off cranially and extended along the colliculi and tela chorioidea to the roof of the third ventricle, which it invaginated ending at the level of the dorsal end of the hippocampus. The volume of the fixed tumor was approximately the same as that in cat A 57-581.

The tumor was connected to the meningeal tissue under the corpus callosum at the level of the infundibulum. In the ventral portion of the tumor, meningeal strands ran along the vena magna cerebri and the choroid plexus of the third ventricle. The chief portion of the growth lay dorsal to the vena magna cerebri in this region (Fig. 6). On the right and left below this vein were sausage-shaped structures which consisted of arterioles with collagen-rich walls. Psammoma bodies were also present here and were invested by a stroma meager in collagen fibers (Fig. 4). The stroma of the choroid plexus contained increased amounts of fibrous connective tissue.

The tumor was poor in collagen, except at the periphery, where somewhat thicker cell layers were suggestive of a capsule. The tumor consisted of small whorls of concentric cell layers and groups of cells with ovoid vesicular nuclei, peripheral concentration of chromatin, and poorly visible cytoplasm (Fig. 7). A few nuclei were pyknotic and karyorrhectic. Concentrically layered calcification was present in the center of some of the whorls. Not infrequently, some club- and spear-shaped calcium deposits were seen in the rather sparsely occurring stroma.

In the region of the anterior colliculus, the tumor was approximately eight times larger in cross section than at the level of the infundibulum, and lay immediately below the corpus callosum, surrounded on both sides by the hippocampus. The tumor was separated from the corpus callosum and hippocampus by a distinct layer of meninx. A large venous vessel, evidently the vena magna cerebri, extended through the dorsal part of the tumor (Fig. 8). The histologic structure of this caudal part was different from that of the other parts just described and was, in fact, different from all of the meningiomas encountered. Most conspicuous was a large number of round and elongated psammoma bodies of greatly diverse size, chiefly concentrically arranged. These bodies were interspersed with large and small, round and irregularly shaped granules and were surrounded by a ring of small spindle-shaped cells. These cells possessed chromatin-rich, as well as vesicular, nuclei and made up a cell structure which was rich in capillaries and contained large vacuoles. The tumor in its cranial position was clearly an endotheliomatous meningioma, but its caudal portion could not be fitted into any known classification (Fig. 9). The more compact endotheliomatous portion extended ventrally into a wedge shape. Periadventitial calcium deposits were visible in many ves-

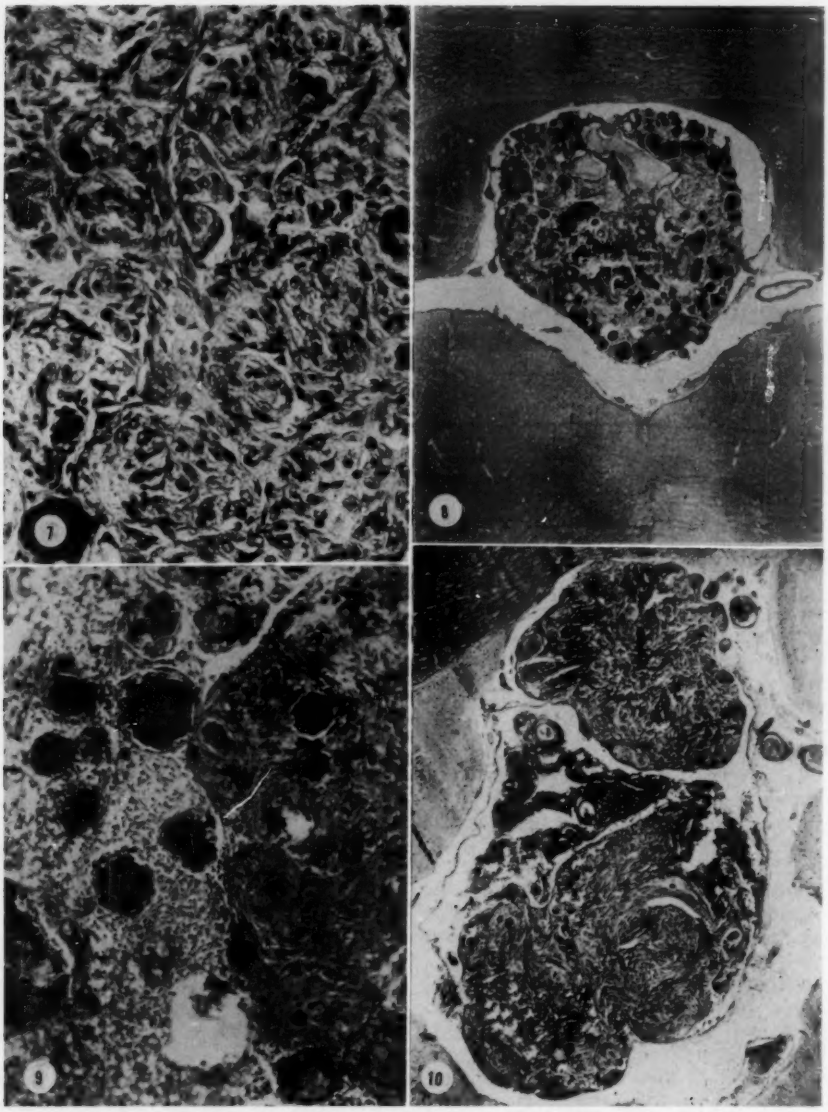


Fig. 7—Small nodules and groups of concentrically laminated cells. The chromatin of the vesicular nuclei is concentrated on the periphery. H & E stain; orig. mag. x 350.

Fig. 8—Unclassified portion of tumor situated over the anterior colliculi, under the corpus callosum and between the left and right hippocampus. The venous vessels run lengthwise through the dorsal part of the tumor. The darker area in the ventral portion of the tumor is the caudal end of the endotheliomatous meningioma. H. & E stain; orig. mag. x 24.

Fig. 9—Unclassified meningioma with numerous calcium granules surrounded by a net of cells (left) and sharply circumscribed endotheliomatous portion of meningioma (right). H & E stain; orig. mag. x 170.

Fig. 10—Meningioma nodules dorsal and ventral to a vein in the caudal part of the tumor. A portion of the corpus callosum is visible above the tumor, and areas of hippocampus to the right and left of it. van Gieson stain; orig. mag. x 30.

sels, mostly arterioles. More rarely, mineral deposits involved the whole of the vessel wall. No morphologic change was seen in the epiphysis, even though it was thoroughly examined by means of serial sections.

Case 3.—A spindle-shaped tumor was found extending from the colliculi to the rostral end of the third ventricle in an 11-year-old spayed female (A 58-38). It was 12 mm. long, and its greatest width, 6 mm., occurred at the level of the infundibulum. This was the largest meningioma of this series. A sausage-shaped structure extended from the caudal end of the tumor along the dorsal and ventral aspects of the venous vessel, which in this area was invested by much collagenous connective tissue (Fig. 10). At the level of the entrance to the aqueduct, a large nodule on one side and several small nodules on the other were united in the center by a fibrotic choroid plexus.

The large tumor nodule consisted of strands and bundles of cells running in various directions. These contained moderately numerous collagen fibers and many long calcium spears which were oriented in the direction of the tissue strands. The calcium deposits were often found pointing in various directions and formed many-faceted structures. Only a few typical whorls, some with psammona granules, were present, and these were at the periphery of the nodule. The nuclei were oval to spindle-shaped and contained somewhat more finely granular chromatin than the vesicular nuclei of the endotheliomatous meningiomas. The nodule, classified as a fibroblastic meningioma with extensive calcification (Fig. 16), was relatively avascular, and many of the precapillaries and capillaries that it did contain had calcified walls.

Among the smaller nodules on the other side of the choroid plexus, the dorsal one was typical of an endotheliomatous meningioma, consisting almost entirely of cell whorls. The centers of many of these whorls contained necrotic groups of cells and calcium granules (Fig. 14). The ventral nodule was of an intermediate character, inasmuch as cell groups with a clear tendency to whorl formation were interspersed with disorganized ones. All three nodules had well-marked zones, especially at their peripheries, of predominantly spindle-shaped cells whose cytoplasm was filled with granules of brown pigment (Fig. 17). These pigment-laden cells were similar to others found in sections of visceral organs and will be the subject of a separate report.

In the cranial part of the third ventricle, the choroid plexus was more villous and had no discernible fibrosis. Finger-shaped meningiomatous processes accompanied the choroid plexus laterally on both sides, extending almost to the region of the foramen of Monro (Fig. 2, 11).

In addition to this meningioma in the third ventricle, various other meningiomatous nodules were found on the corpus callosum and along the median fissure, some grossly and others only microscopically visible (Fig. 13). These consisted of typical cell whorls with chromatin-poor vesicular nuclei and degenerated centers. About half of the whorls contained psammoma bodies. The leptomeninx over the cerebral hemispheres was loosened. Heaps of arachnoid cells were found, more in the sulci than on the gyri. Some of these were compact cell clusters, but some of the heaps had distinct whorl structure with psammoma granules, and can probably be considered early stages of meningiomas, arising from the arachnoid cells (Fig. 18). As the description indicates, several small meningiomas, histologically of diverse types, were concurrently present.

Case 4.—Numerous small nodules on both sides of the choroid plexus, along the venous vessel at the level of the chiasma, were rich in stroma, poor in vessels, and had only a slight tendency to whorl formation. These growths were classified as fibroblastic meningiomas.

Numerous microscopic nodules were distributed in the arachnoid space over the convexity of the cerebral hemispheres. These had a tendency to whorl formation and contained varying amounts of stroma. An occasional whorl contained psammoma bodies.

A grossly visible fibroblastic meningioma was present in the leptomeninx of the tela choroidea of the fourth ventricle. This was the only cat in which a tumor of this size occurred in this location. In the medial part of the tumor, the cells tended to form onion skin layers, whereas in the lateral part they occurred in bundles and strands (Fig. 19). Intensive calcification occurred here. The nuclei were ovoid, vesicular, and poor in chromatin. A band of collagenous

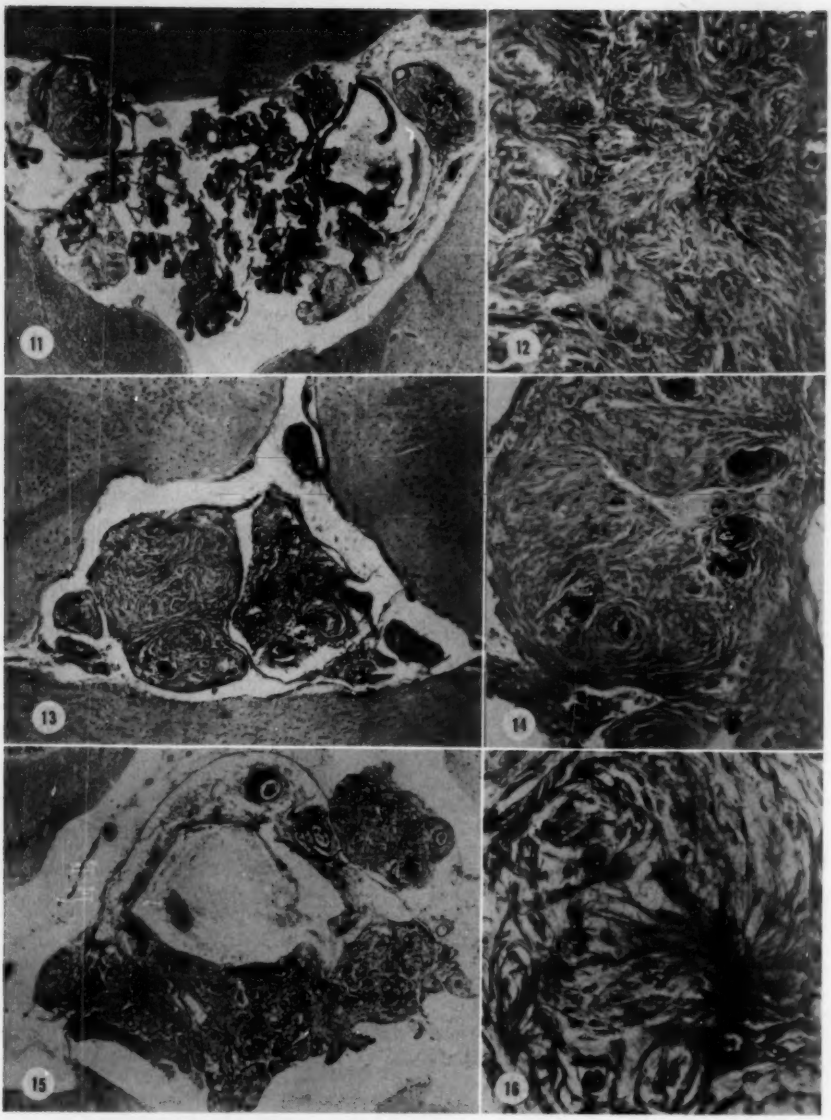


Fig. 11—The choroid plexus of the third ventricle is accompanied laterally on both sides by finger-shaped processes of meningioma (see also Fig. 2). van Gieson stain; orig. mag. x 35.

Fig. 12—Typical cells in small, irregular groups and strands. H & E stain; orig. mag. x 160.

Fig. 13—Small meningioma above the corpus callosum, growing along the blood vessels. Pressure atrophy in the cortex of the cingulate gyrus. Mixed endotheliomatous and fibromatous type. van Gieson stain; orig. mag. x 57.

Fig. 14—Part of an endotheliomatous nodule which contained many nodules, some with psammoma bodies. H & E stain; orig. mag. x 145.

Fig. 15—Nodules of mixed endotheliomatous and fibroblastic type of meningioma. These are located on both sides of the plexus of the third ventricle in the arachnoid space and under the pia on one side. van Gieson stain; orig. mag. x 38.

Fig. 16—Fibroblastic meningioma with heavy deposits of calcium needles. van Gieson stain; orig. mag. x 350.

fibers, thick in some areas, ran between the meningioma and the choroid plexus.

Case 5.—In a 15-year-old spayed female (A 58-90), two small nodules, 3 mm. and 1 mm. in diameter, were found in the choroid plexus between Ammon's horn and the roof of the third ventricle, at the level of the infundibulum. The caudal portion of the plexus and the epiphysis were not involved by neoplasm. The nodules were areolar in histologic structure, consisting of cell bundles oriented in various directions. There were numerous small whorls, consisting of few cells. Many of the whorls were calcified or else had definite central lumens containing some erythrocytes and giving the tumor a more richly vascular appearance than the other meningiomas described herein.

These tumor nodules were classified as mixed fibroblastic and endotheliomatous meningiomas.

Case 6.—A tumor of microscopic size was located immediately below the corpus callosum, between the pia mater and the vena magna cerebri, at the level of the anterior colliculi in a 10-year-old castrated male cat (A 58-59). The arachnoid cell nodule of concentrically layered, densely packed cells with ovoid vesicular nuclei was connected to the pia mater by delicate strands of tissue. There were spindle-shaped meningeal proliferations on both sides of the epiphysis and the vena magna cerebri involving the choroid plexus of the third ventricle as far as its rostral third. They reached their greatest diameter, 1.5 to 2.0 mm., at the level of the infundibulum. Both structures were in the arachnoidal space, *i.e.* they were lateral to the pia mater, medio-ventral to the plexus stroma, and medio-dorsal to the vena magna cerebri. A third nodule, 2 mm. in diameter and extending under the pia mater, *i.e.*, between it and the hippocampus, was found at the level of the infundibulum (Fig. 13).

The two spindle-shaped structures consisted almost exclusively of whorls, which were partially calcified in their centers. The sparse stroma contained multishaped forms of calcium spears and clubs.

The third above-mentioned nodule had a lesser tendency to whorl formation and had an abundant stroma. The cells and fibers were irregularly arranged in all directions.

This tumor, consisting of the three nodules, was classified as a mixed endotheliomatous and fibroblastic meningioma.

Case 7.—Cat A 58-100 was a castrated male, 8 years old. A meningeal proliferation along the choroid plexus on both sides of the epiphysis was situated in the region of the foramen of Monro, immediately below the fornix in close apposition to the meningeal covering of the ventricular roof and above the choroid plexus. The transverse dimension of the mass was 3.5 mm., and the dorsoventral dimension was 2.5 mm.

Microscopically, the tumor had distinct whorl formation of typical meningothelial cells. In addition, these cells were arranged in groups and bundles that ran in all directions. Calcium concretions were present in capillaries and in small whorls.

This tumor was considered to be a mixed endotheliomatous and fibroblastic meningioma.

Case 8.—A microscopic meningioma was found in a spayed, 2-year-old cat (A 58-178). It was in the arachnoid space overlying the cingulate gyrus, and consisted of one whorl of concentrically layered cells. The nuclei were vesicular, poor in chromatin, and had a distinct nuclear membrane. The center of the whorl had regressive cellular alterations in the form of pyknotic and fragmented nuclei and eosinophilic, shrunken cytoplasm. The surrounding arachnoid was rich in cells (Fig. 18).

This tumor was considered as an early stage of an endotheliomatous meningioma.

Discussion

One or more meningiomas were found in eight of 155 cat brains examined routinely at necropsy. Tumors of neuroectodermal origin were not found. None of the meningiomas had elicited tangible clinical signs, even though small areas of compression atrophy of the adjacent brain tissue was demonstrable in several cases. The large number of meningiomas found in relation to the number of brains examined raises several questions: *viz.*, whether such tumors are usually overlooked, whether cat brains in general are carefully examined, or whether the occurrence of this tumor was unusually frequent in the vicinity of Boston.

It is understandable that tumors of small size, such as most of these described

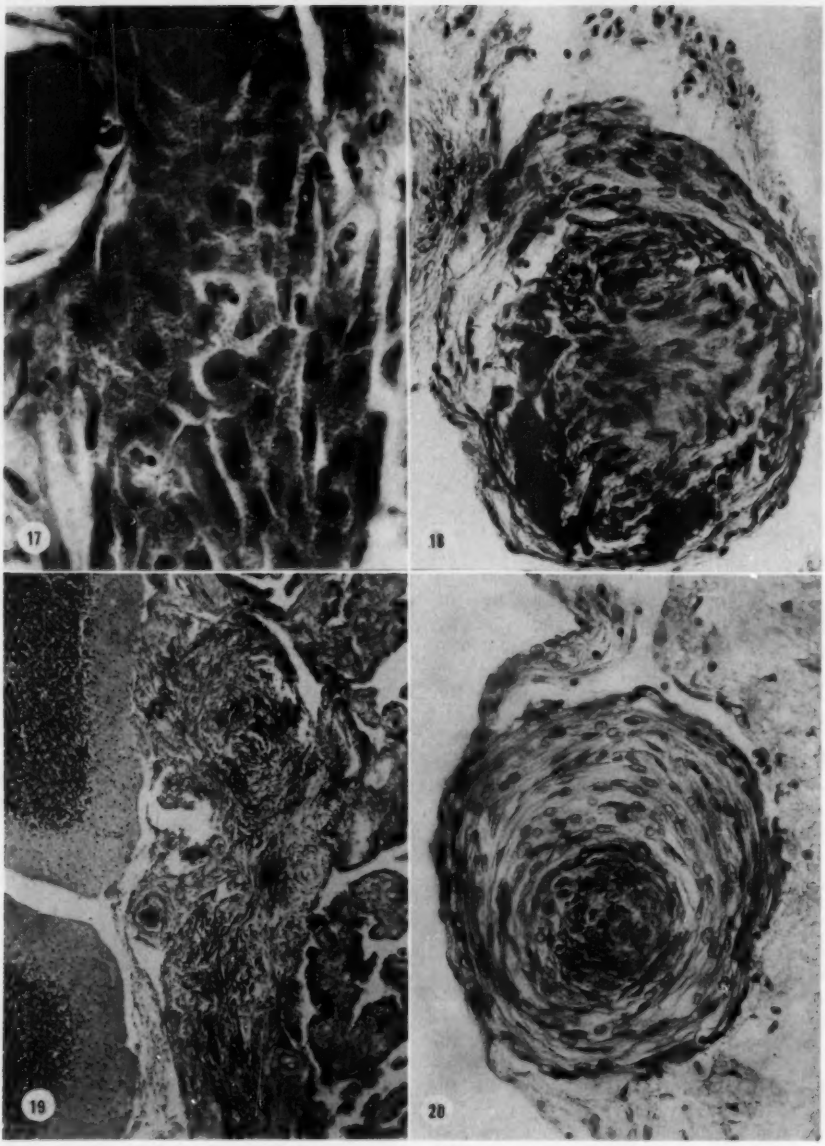


Fig. 17—Zone adjacent to a meningioma with spindle-shaped cells that are filled with granules of brown pigment. H & E stain; orig. mag. x 800.

Fig. 18—Small meningeal proliferation in the arachnoid space above a cerebral hemisphere. H & E stain; orig. mag. x 350.

Fig. 19—Fibroblastic meningioma in the tela choroidea of the plexus of the fourth ventricle. (The medial side is at the top of the picture, the lateral side is below.) H & E stain; orig. mag. x 70.

Fig. 20—Early stage of an endotheliomatous meningioma in the arachnoid space above the cingulate gyrus. H & E stain; orig. mag. x 350.

herein, would remain undetected if the brain were not examined systematically and carefully. This is particularly true since the tumors had a color almost indistinguishable from their surroundings, and many of those which are grossly visible lay close to the pineal body and could easily be mistaken for it. It is far more peculiar that none of these meningiomas attained a size large enough to evoke clinical signs. Presumably, such tumors could be found even with a more casual examination of the brain. The affected cats often reached an advanced age and died of natural causes, which presumably gave the tumors time enough to attain a larger growth if they were going to do so. There is also the question of the eventual fate of these meningiomas, in the event that the life of the host had extended longer. Even with the support of histologic studies, it appears futile to speculate on whether the tumors would remain small and clinically silent, or whether they would develop into large, space-occupying growths. However, further studies at the Angell Memorial Animal Hospital indicate that meningiomas may produce clinically recognizable signs.*

Nearly all the meningiomas found were poorly vascularized, and the sparsely occurring vessels in some cases had undergone regressive changes, chiefly calcification, which evidently choked off the blood supply and thereby retarded growth. The anatomic location of most of the meningiomas in our series is worthy of note. In seven of the eight cases (*i.e.*, in all of the old cats) a meningeal proliferation was found under the corpus callosum; as already mentioned, it protruded into the third ventricle. According to the voluminous literature, this locus is rare in human beings.[4,7] That a meningioma can occur above the choroid plexus of the third ventricle, or in its stroma, is readily understandable, since part of the pia mater together with the choroid plexus skirts along the corpus callosum and Ammon's horn and forms the means of suspension of the choroid in the ventricle. The stroma of the choroid plexus is a leptomeningeal extension and is thus in a position to originate meningeal tumors.

It appears extraordinary that the meningiomas accompanying the choroid plexus of the third ventricle are not exclusively of the fibroblastic type which, according to the literature, is the case in man.[4,7,10,11] The present cases were more variable and pleomorphic. In individual nodules, the typical cells occurred almost exclusively syncytially, united in concentrically laminated arrangements. In growths with many whorls, the reticulum fibers, as demonstrated by Wilder's reticulin stain, were restricted to the stroma. Such tumors were associated with the endotheliomatous type of meningioma.

In five of the seven cases of tumors with leptomeningeal extension, the meningiomas or at least parts thereof were of the mixed endotheliomatous and fibroblastic type. Apart from whorls, the endotheliomatous cells also occurred in irregular groups and in strands and striae which ran in all directions.

In two cases of meningeal proliferation, the growth was predominantly of the fibroblastic meningioma type. There was an absence of typical cells and of whorl formation. One such tumor, in which the meningioma extended into the third ventricle and median fissure, had islands of coarsely vacuolated fat cells.

Thus, this report shows that not only fibroblastic but also other histologic types of meningiomas can arise in the leptomeningeal processes (tela choroidea, plexus stroma) of the cat. It also confirms Russell's assertion that most meningiomas contain transition forms to other types, and that more than one histologic form is visible in the majority of them. Henschen [3] also states that it is more difficult to create a strict classification of the meningiomas, since the microscopic appearance of a single tumor not infrequently varies from section to section. In general, most of the tumors reported here could not be organized into any particular system. In an altogether arbitrary classification, one is readily prone simply to pass over whatever does not fit into that particular system.

The meningiomas classified as endotheliomatous type also had single or multiple psammoma bodies, in which a concentric layering was discernible. Independent of psammoma bodies, more or less extensive calcification occurred in all of the meningiomas. These club-shaped and spear-shaped mineral deposits appeared, on the basis of Wilder's stain, to affect chiefly the reticulum fibers.

* Jones, T. C.; Angell Memorial Animal Hospital, Boston, Mass.: Personal communication.

In two of the seven meningiomas accompanying the choroid plexus of the third ventricle, there were also meningeal proliferations above the corpus callosum and in the median fissure; in two cases, there were numerous small nodules on the convexity of the cerebral hemispheres. These tumors were histologically as pleomorphic as the ones discussed previously. As a further exception, there was also one small fibroblastic meningioma in the stroma of the choroid plexus of the fourth ventricle. However, no meningeal proliferation of the plexus stroma of a lateral ventricle was found.

The 7 cats with meningioma of the tela choroidea of the third ventricle were 8 to 17 years old. With one exception we were thus dealing exclusively with old or very old cats. One cat was male, 3 were castrated males, and 3 were spayed females.

References

[1] Cooper, E. R. A., and Howarth, I.: Some Pathological Changes in the Cat Brain. J. Comp. Path., 66, (1956): 35–38.

[2] Dahme, E.: Meningiome bei Fleischfressern. Berl. Münch. tierärztl. Wchnschr., 70, (1957): 32–34.

[3] Henschen, F.: Tumoren des Zentralnervensystems und seiner Hüllen. In *Handbuch der Speziellen Pathologischen Anatomie und Histologie*. Vol. 13. Part 3, Springer-Verlag, Berlin, 1955.

[4] Kernohan, S. W., and Sayre, G. P.: Tumors of the Central Nervous System (Section X, Fascicles 35 and 37). In *Atlas of Tumor Pathology*, AFIP, Washington, D.C., 1952.

[5] McGrath, J. T.: *Neurologic Examination of the Dog*. 2nd ed. Lea & Febiger, Philadelphia, (1960): 152.

[6] Monteagudo, O. G., and Purpura, D. P.: Epileptogenic Effects of an Auditory Cortex Meningioma in a Cat. Cornell Vet., 49, (1959): 374–379.

[7] Russell, D. S., and Rubinstein, L. J.: *The Pathology of Tumors of the Nervous System*. Edward Arnold Ltd., London, 1959.

[8] Smith, H. A., and Jones, T. C.: *Veterinary Pathology*. Lea & Febiger, Philadelphia, (1957): 208.

[9] Weed, L. H.: The Cells of the Arachnoid. Johns Hopkins Hospital, Bulletin 31, (1920): 343–350.

[10] Zulch, K. J.: *Die Hirngeschwulste*. Johann Abrosius Barth Verlag, Leipzig, 1956.

[11] Zulch, K. J.: Biologie und Pathologie der Hirngeschwulste. In *Handbuch der Neurochiurgie*. Vol. 3, Springer-Verlag, Heidelberg, 1956.

SUMMARIO IN INTERLINGUA

Studios in Meningiomas in Cattos

Un o plure meningiomas esseva trovate in octo inter 155 cerebros felin que esseva cautemente examinate al necropsia. In 7 cattos, de etates de inter 8 e 17 annos, le tumores esseva in le tela choroidee del tertie ventriculo. Histologicamente illos esseva diverse in character. Esseva diagnosticate variantes endotheliomatose, psammomatose, fibroblastic, e endotheliomato-fibroblastic mixte.

In duo del cerebros, proliferationes meningee super le corpore callose esseva notate a parte le meningiomas que se extendeva ad in le tertie ventriculo. In duo altere casos, numerose micre nodulos esseva presente super le convexitate del hemispherios cerebral.

Es emphatisate le valor de examines systematic del cerebros de omne animales presentate al necropsia.

Contrast Encephalography and Ventriculography in the Dog—Preliminary Studies

B. F. Hoerlein, D.V.M., Ph.D.; M. F. Petty

SUMMARY

In preliminary studies, techniques were devised so that pneumoencephalography, either by the cisternal or the lumbar route of administration, could be performed in the dog but results were inconsistent. Air injected directly into ventricles does not at the present time seem to be a practical procedure in ventriculography. However, radiopaque contrast mediums, such as ethyl iodophenylundecylate and n-propyl ester of 3:5 diiodo-4-pyridone-n-acetic acid, do offer a great deal of promise for canine ventriculography.

INTRODUCTION

Pneumoencephalography and ventriculography in man have become commonplace diagnostic tools. These techniques are used in diagnosing a variety of brain lesions such as internal hydrocephalus, tumors, abscesses, blood clots, and other conditions that change the position or size of the ventricles of the brain. Because such procedures applied to dogs would greatly aid in the diagnosis of brain diseases, the object of this investigation was to devise a method of roentgenographic visualization of the brain, particularly the ventricles. The techniques, if adapted for dogs, would provide the basis for clinical brain surgery in that species.

Review of Literature

There is little existing literature on ventriculography and pneumoencephalography in the dog but a great deal pertaining to man. Ventriculography and encephalography were employed as early as 1918 by Dandy,[1] who suggested that air as a medium would be nonirritating, nontoxic, and readily absorbed and excreted. Dandy suggested that the introduction of air into the lateral ventricles be called ventriculography. The introduction of air into the spinal subarachnoid space, in such a way that as the air ascends in the spinal canal it then enters the various spaces over the cerebral hemisphere and also the ventricles, is termed encephalography. Various mediums, particularly air and oxygen,[1] have been used in so-called pneumoencephalography.

According to Robertson,[6] air is much more slowly absorbed than oxygen. Oxygen from the subarachnoid spaces may be too rapidly absorbed by the anoxic blood. Air is considered to be better for use in the ventricles of infants, but oxygen might be better in larger areas or ventricles. When 20 to 25 cc. of air are slowly injected into the spinal subarachnoid space, there seems to be little after effect; but when this quantity is exceeded, headaches may occur. It is standard practice to change the position of the head as the medium is being given, so as to outline the various areas of the ventricles more adequately. Robertson [6] claimed that head position must vary in order to demonstrate the various parts of the ventricular system. When the head and neck are fully flexed, the cisterna magna, the fourth ventricle, and the cerebral aqueduct are more fully visualized. At a 45-degree angle, more gas goes into the fourth and third ventricles. At a 25-degree angle, more gas passes into the third ventricle, and at a 10 to 20-degree angle, most of the gas passes into the third and the lateral ventricles.

Many authorities feel that encephalography is more dangerous than ventriculography, particularly in cases of increased intracranial pressure. It has been found, however, that the various discomforts of pneumoencephalography, such as headaches and shock, can be relieved by inhalation of oxygen during and following the completion of the procedures. Moreover, it has been demonstrated that in a considerable number of cases it is impossible to fill the ventricles satisfactorily by the injection of air into the spinal subarachnoid space. Just why this is true, particularly in patients without intracranial lesions is not known. The subarachnoid spaces may be outlined in perfect detail, but the failure of the ventricles to fill makes it necessary to perform a ventriculogram. It is perhaps for this reason that ventriculography

Received for publication Jan. 23, 1961.

Publication No. 880, School of Veterinary Medicine, Auburn University, Auburn, Alabama. Dr. Hoerlein is head, Department of Small Animal Medicine and Surgery; Mr. Petty is a student fellow (class of 1962) of the Mark L. Morris Animal Foundation, Denver, Colo.

This project was supported by the Mark L. Morris Animal Foundation, Denver, Colo., in cooperation with the Scott Research Foundation, Auburn, Ala.

The authors thank Dr. T. C. Fitzgerald, Department of Anatomy and Histology, and Dr. A. H. Groth, Jr., Department of Pathology and Parasitology, Auburn University, for their help and consultation.

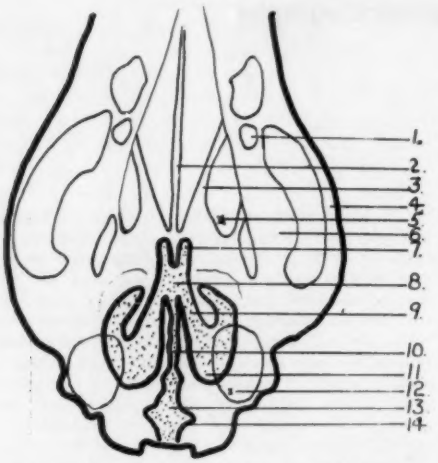


Fig. 1—The relations of the ventricles to other parts of the skull, dorsoventral view. (1) molar tooth, (2) nasal septum, (3) maxillary bone, (4) zygamatic arch, (5) frontal sinus, (6) coronoid process of mandible, (7) anterior horn of lateral ventricle, (8) interventricular foramen, (9) body of lateral ventricle, (10) suprapineal process of 3rd ventricle, (11) inferior horn of lateral ventricle, (12) petrous temporal bone, (13) 4th ventricle, (14) lateral recess of 4th ventricle.

is more frequently used at the present time than encephalography.[2]

Ventriculography requires a surgical procedure in which the head is shaved and small incisions are made in the scalp at the level of the external occipital protuberance. Burr holes are then made in the cranial vault and the dura mater is exposed. After the dura is incised, a needle is inserted into the lateral ventricle. The ventricular fluid is drained and air is inserted in order to properly visualize the ventricles.[7] This procedure usually requires general anesthesia, whereas pneumoencephalography does not.

Some work has been done with contrast mediums other than air. Ethyl iodophenylundecylate* has been used via the lumbar route by Mones[5] and via the ventricular route by Horwitz.[4] Horwitz suggests using 1.5 to 2.0 cc. of ethyl iodophenylundecylate in the lateral ventricles. Mones suggests using 6.0 to 9.0 cc. of this medium in the lumbar subarachnoid space. When the needle is removed, the patient is placed in a dorsal recumbent position with the neck fully flexed. The table is tilted with the patient's head at 80 to 90 degrees for one minute. The patient is brought to a 70-degree tilt, and lateral and anterior-posterior radiographs are taken in this position. Then the person is placed in the erect position for five minutes, and the medium is removed from the lumbar region by means of another puncture. In nine of 14 patients, the fourth ventricle was successfully filled. This

* Pantopaque, produced by Lafayette Pharmacal Inc., Lafayette, Indiana.

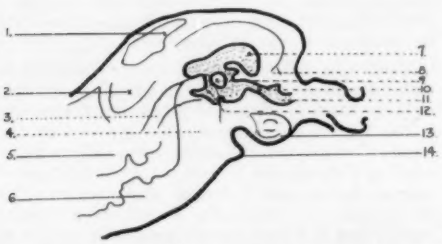


Fig. 2—The relations of the ventricles to other parts of the skull, lateral view. (1) Frontal sinus, (2) orbit, (3) zygomatic arch, (4) coronoid process of the mandible, (5) upper molar teeth, (6) lower molar teeth, (7) lateral ventricle, (8) internal occipital protuberance, (9) thalamus (mass intermedia), (10) cerebral aqueduct, (11) 4th ventricle, (12) ventral part of 3rd ventricle, (13) bulla ossea, (14) angular process of the mandible.

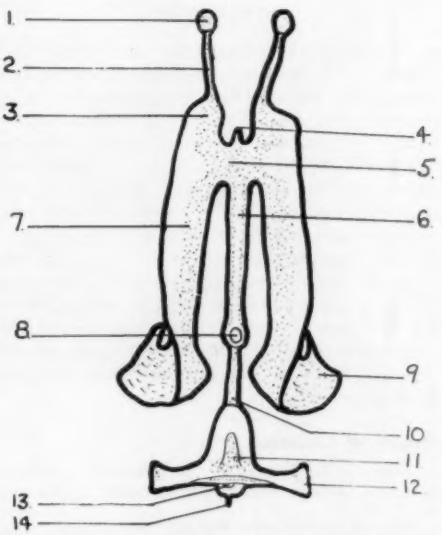


Fig. 3—Sketch of the lateral ventricles of the dog, dorsoventral view. (1) Cavity of olfactory bulb, (2) olfactory stem, (3) anterior horn of lateral ventricle, (4) 3rd ventricle, (5) interventricular foramen, (6) 3rd ventricle, (7) body of the lateral ventricle, (8) suprapineal process of 3rd ventricle, (9) inferior horn of lateral ventricle, (10)) cerebral aqueduct, (11) anterior part of 4th ventricle, (12) lateral recess of 4th ventricle, (13) posterior part of 4th ventricle, (14) process which extends into central canal of medulla oblongata.

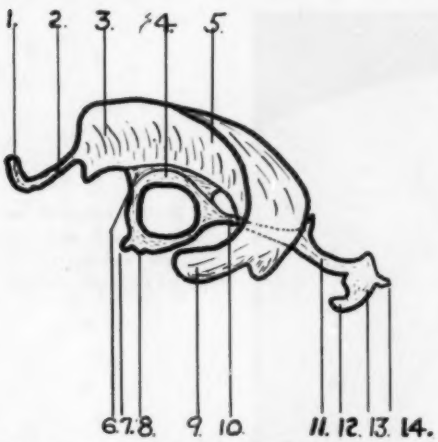


Fig. 4—Sketch of the lateral ventricles of the dog, lateral view. (1) Cavity of olfactory bulb, (2) olfactory tract, (3) anterior horn of lateral ventricle, (4) 3rd ventricle, (5) body of lateral ventricle, (6) interventricular foramen, (7) optic recess of 3rd ventricle, (8) infundibular part of 3rd ventricle, (9) inferior horn of lateral ventricle, (10) cerebral aqueduct, (11) 4th ventricle, (12) lateral recess of 4th ventricle, (13) posterior part of 4th ventricle, (14) process extending into the central canal of the medulla oblongata.

procedure is called rhomboencephalography. Mones feels that the use of ethyl iodophenylundecylate in the brain ventricles is a relatively safe procedure,

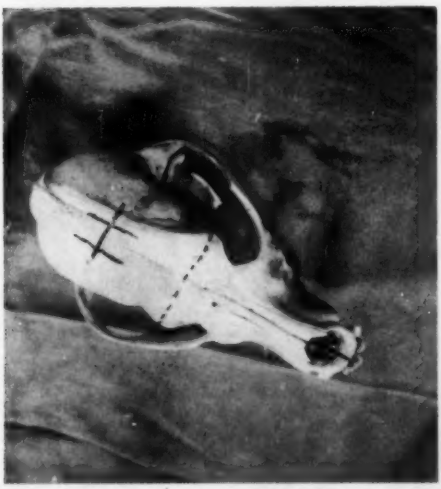


Fig. 5—Landmarks for ventricular tap. A line drawn midway between lateral canthus of the eye to the external occipital protuberance, 1 cm. from the midline.

and that it creates little or no reaction.[5] This, however, is not true of other mediums, particularly thorium dioxide.[6]

Procedures and Results

Anatomy.—Since one of the main purposes of this study was centered around the demonstration of the brain ventricles, it was important to review the anatomy of the ventricles in the dog. An article describing the form and relations of the ventricles has recently been published by Fitzgerald.[3] The fourth ventricle is commonly known as the cavity of the hindbrain and is situated between the central canal of the spinal cord and the third ventricle anteriorly. Anteriorly, it lies above the pons and the anterior part of the medulla oblongata. Laterally, it is bounded by the restiform body and the anterior peduncle of the cerebellum. The posterior portion of the fourth ventricle has free ends which are called the lateral recesses and these are flared like fish tails. The lateral recesses of the fourth ventricle communicate with the subarachnoid space by lateral apertures called the foramina of Luschka. The medial communication with the subarachnoid space is called Magendie's foramen.

The third ventricle is a median unpaired space situated within the diencephalon. This ventricle is a narrow cleft somewhat oval in shape as viewed laterally. The roof is the widest part of the circumference. The floor is the narrowest and is bounded chiefly by the structures which form the hypothalamus. Somewhat anteriorly, the third ventricle communicates with the two lateral ventricles by means of interven-

Fig. 6—Incision site for ventriculography.

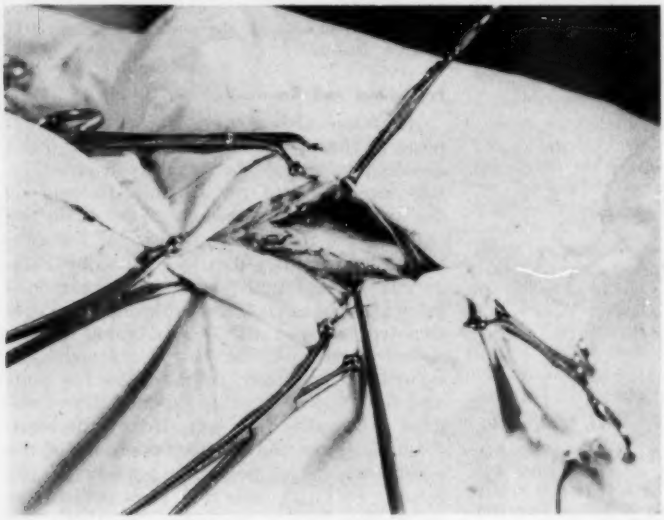


Fig. 7—The temporal fascia is incised and the temporal musculature is "peeled off" the cranium.

tricular foramina. The two lateral ventricles are irregularly shaped cavities located in each cerebral hemisphere and are separated from each other by the septum pellucidum. Each lateral ventricle is divided into three parts: the body, an anterior horn, and a ventral horn. The lateral ventricles occupy quite an extensive area in the canine brain (Fig. 1–4).[3]

In order to study in more detail the ventricles of the canine brain, two dogs were killed. Their heads were placed in a deep freezer until completely frozen; they were then cut in 1 centimeter sections. One head was cut sagitally and the other transversally. It was found that the medial surface of the body of the lateral ventricle might be situated as close as 0.9 cm. from the midline. The lateral ventricle is normally thicker in its posterior part. Measuring

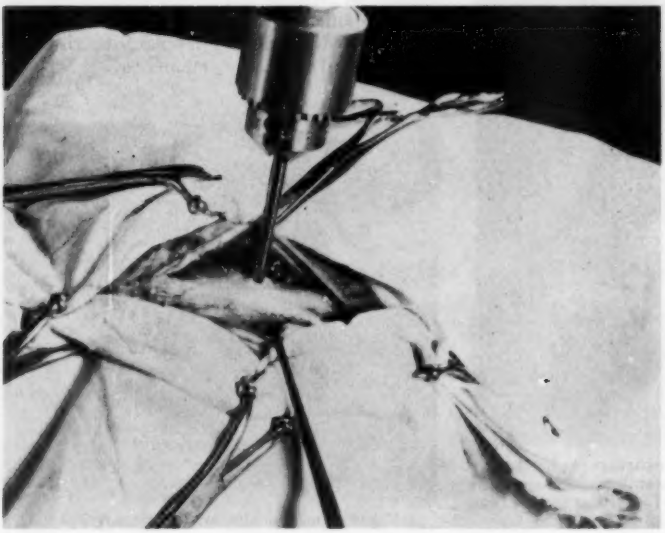


Fig. 8—Drilling a hole for ventriculography.

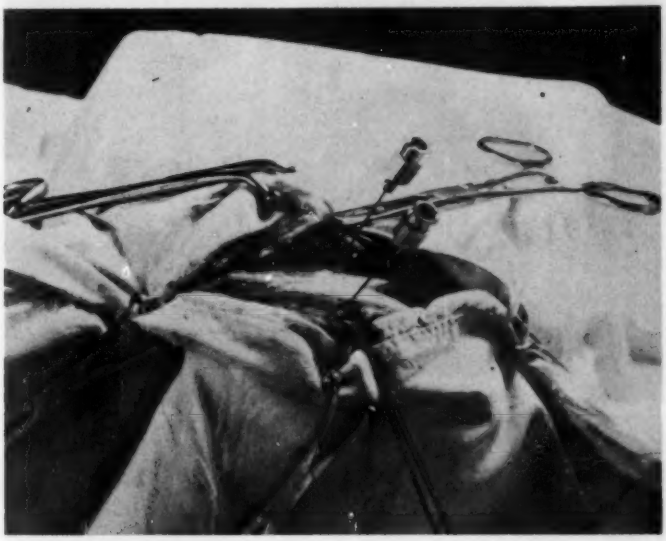


Fig. 9—The needles are introduced in each lateral ventricle.

vertically from 1.0 cm. lateral to the midline, the lateral ventricle is found to be 2.6 cm. beneath the skull. However, the size, depth, and location varied considerably with the breed of dog examined (Fig. 11, 12, 19).

Ventriculography.—As a result of the anatomical studies, it was felt that numerous trials should be performed on a living specimen in order to determine the most convenient and accurate location for a ventricular tap. After the head of the anesthetized dog had been clipped and shaved for surgery, a midline incision of 2.0 to 3.0 inches was made through the skin covering the cranial vault (Fig. 5, 6). The subcutaneous tissue and the fascia immediately adherent to the temporal muscles

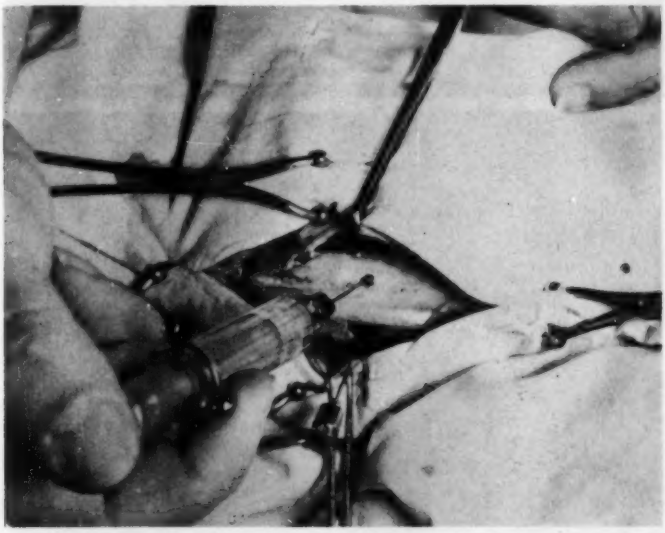


Fig. 10—The contrast medium is introduced into the lateral ventricle.

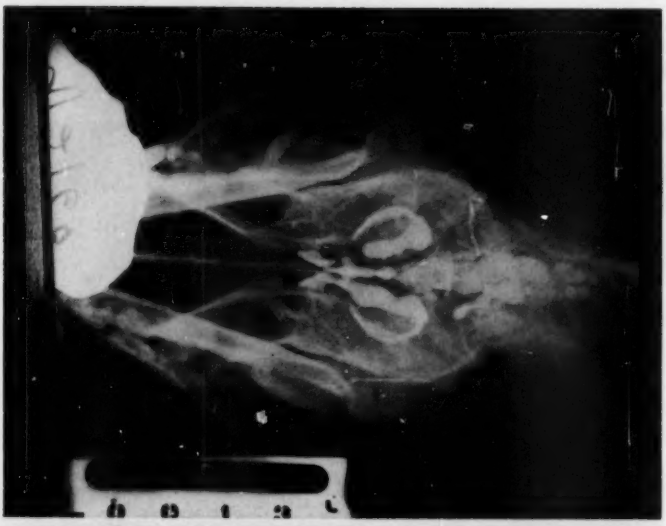


Fig. 11—A dorsoventral ventriculogram, using 3 cc. of ethyl iodophenylundecylate (dog 13).

Fig. 12—A lateral ventriculogram, using 3 cc. of ethyl iodophenylundecylate (dog 13).

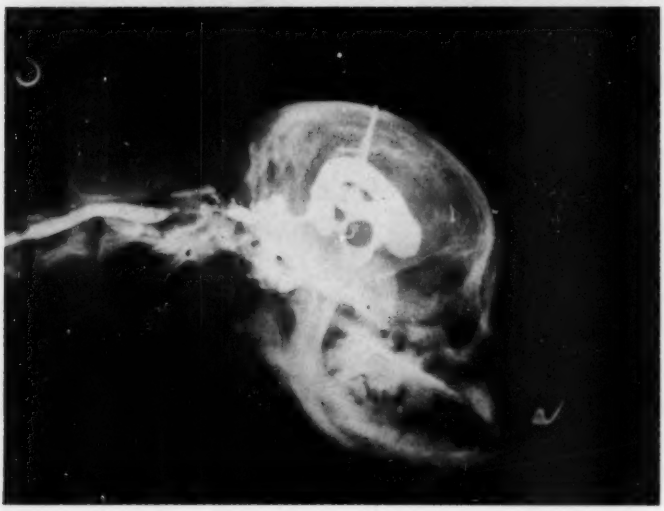


Fig. 13—Ventriculogram, using 3 cc. of ethyl iodophenylundecylate (clinical case, 61-902) Notice the enlarged size.

were incised. It was found that the extent and size of the temporal muscles varied a great deal with the type dog. The temporal muscles were carefully pealed away from the cranium from 0.5 to 1.0 inch from the midline (Fig. 7). After several trials, it seemed that a midpoint between the lateral canthus of the eye and the external occipital protuberance was a good landmark.* A trochar-pointed intramedullary pin fastened to a Jacob's hand chuck was then used to drill a small hole approximately 0.5 cm. from the above landmark (Fig. 8). After several trials with various sizes of needles and pins, it was decided that the conventional Quincke 22-gauge 3-inch spinal needle would be a satisfactory instrument to insert into the lateral ventricles (Fig. 9). When this needle was used, it was found that the tunnel drilled into the cranium should be very small. The needle can then be forced slightly through the hole. It will fit snugly in the cranial tunnel, will not penetrate the ventricle too abruptly, and will remain much more stationary for an accurate puncture. The needle was then directed ventrally in an effort to keep it the same distance from the midline as it was inserted. The stylus of the needle was frequently removed, and careful observation was made for the slowly ascending ventricular fluid. When both ventricles had been properly punctured, various contrast mediums were used for radiographic procedures (Fig. 10). After the materials were administered, the ventricular needles were withdrawn and the temporal fascia was sutured. It was found that no more temporal muscle should be removed from the cranium than necessary so that hemorrhage could be more easily controlled. The suturing of the skin over the temporal fascia was routine and the head was generally bandaged postoperatively.

After the contrast medium had been administered, various radiographs were made of the head in different positions. When the left ventricle was injected, the head was immediately tilted downward in order to visualize the anterior portion of the lateral ventricle and then was tilted upward so that the medium flowed to the posterior portion of the left lateral ventricle. Then the animal was placed in the right recumbent position in order for the medium to flow to the right lateral ventricle, and the same changes in positioning were made with each radiograph. If this was done properly, less contrast medium was needed to demonstrate the size and position of the ventricles.

The contrast mediums used, besides ethyl iodophenylundecylate, were diathrizoate sodium,* diatrizoate methylglucamine,** methylglucamine diatrizoate,† sodium acetrizoate,‡ and n-propyl ester of 3:5 diiodo-4-pyridone-n-acetic acid.§ All but ethyl iodophenylundecylate and the last medium mentioned were so toxic that the animals died with two to five hours after administration. However, ethyl iodophenylundecylate and n-propyl ester of 3:5 diiodo-4-

* This injection site had been suggested by Dr. William Gay, National Institutes of Health, Bethesda, Md.

* Hypaque 50%, produced by Winthrop Laboratories, New York, N.Y.

** Cardiographin, produced by Squibb, E. R. & Sons, New York, N.Y.

† Renographin, produced by Squibb, E. R. & Sons, New York, N.Y.

‡ Urokon, produced by Mallinckrodt Chemical Works, St. Louis, Mo.

§ Dionosil, produced by Glaxa Lab., Ltd., Greenford, England.

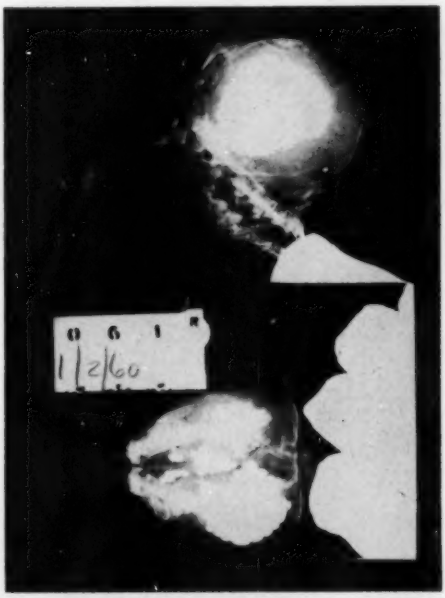


Fig. 14—Internal hydrocephalus in a 2-month-old Pekingese. Ethyl iodophenylundecylate (9 cc.) was introduced in the lateral ventricles after the animal had died. Droplet formations are due to cold tissues.

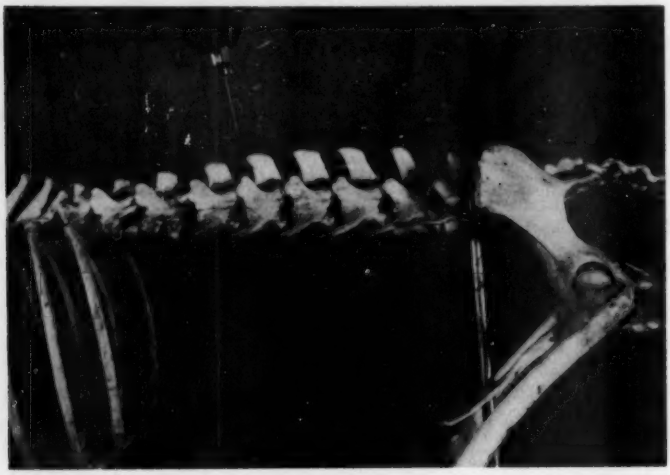


Fig. 15—Skeletal landmarks for lumbar pneumoencephalography.

pyridone-n-acetic acid produced no visible clinical signs, and very little tissue reaction was seen on histopathologic examination. It was felt, because of its flow characteristics, that ethyl iodophenylundecylate was the best medium for use. The amount of this medium administered varied from 1 to 3 cc.

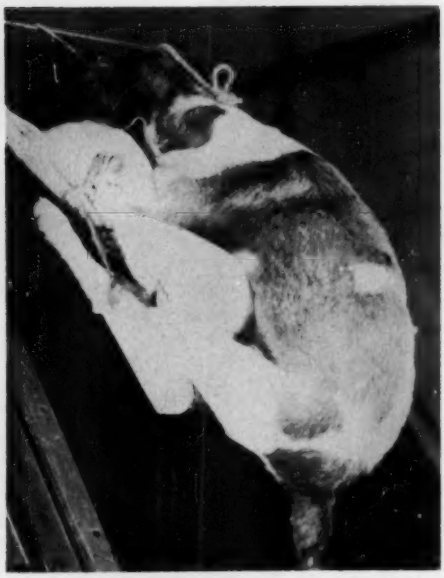


Fig. 16—The position of the dog for lumbar pneumoencephalography.

Air was administered as a contrast medium in 3 dogs, and poor radiographic contrast was observed. Since the ventricles of the dog were observed to be small and air tended to leak out around the sides of the needle, air was not a satisfactory medium in most cases. Also, since the petrous temporal bone was in the same transverse place as the lateral ventricles, visibility of the ventricles in the radiograph was poor (Table 1).

Pneumoencephalography, Lumbar Route.—Pneumoencephalography was tried in 16 dogs. In 8 of them, air was injected by the lumbar route; in 8 air was injected into the cisterna magna. In lumbar pneumoencephalography, an area of skin was prepared over the first or second lumbar interspaces for an aseptic tap with a 22-gauge, 3-inch, Quincke spinal tap needle (Fig. 15). The dog was then anesthetized with pentobarbital sodium and tilted in a 70 to 80-degree angle in a sternal recumbent position (head upwards). His hind legs were drawn forward and tied to the front legs so that a proper arch of the spinal lumbar segment was obtained (Fig. 16). The spinal needle was then inserted along the anterior border of the dorsal spine, usually between the second and third vertebrae. As the needle touched the dorsal lamina of the vertebra, its position was varied until the intervertebral space was felt. The needle was then inserted until the dura was contacted and a superficial "flinch" was observed. The stylus of the needle was carefully removed

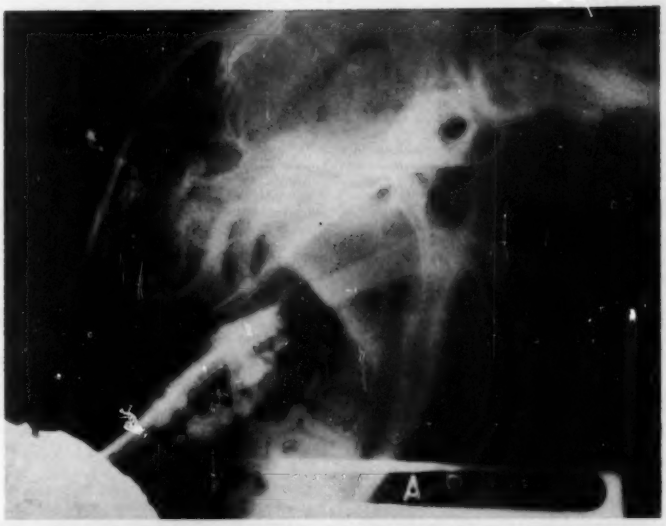


Fig. 17—Lumbar pneumoencephalography (dog 13). Notice the size of the lateral ventricles as compared with the ethyl iodophenylundecylate ventriculogram seen in Figure 12.

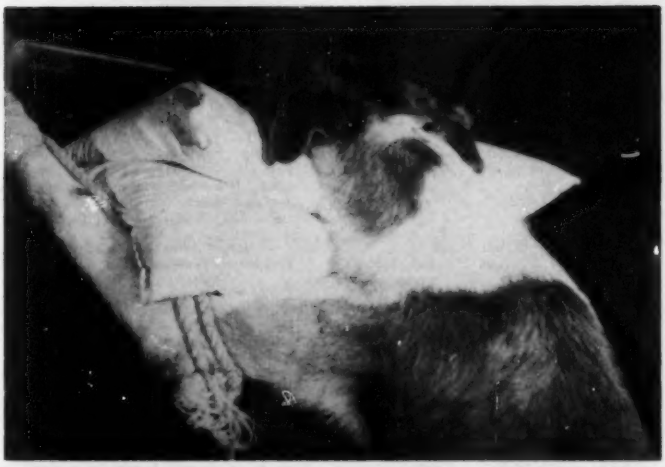


Fig. 18—The position of dog cisternal pneumoencephalography. Notice the needle in the cisterna magna at the atlantooccipital junction.

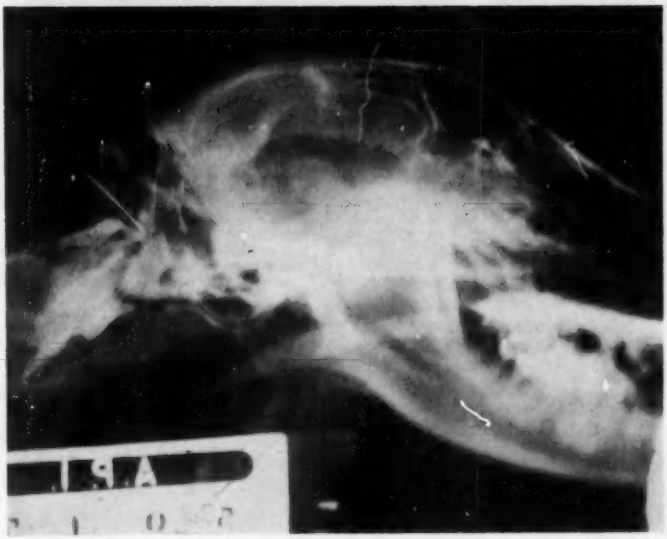


Fig. 19—Cisternal pneumoencephalogram, lateral view (dog 15).

TABLE 1—Results of Ventriculography by Using Several Contrast Mediums

Dog No.	Description	Contrast	Method	Results
1	42 lb. 2 yr. old	Air	50 cc. total given intermittently in each lateral ventricle. Apnea occurred for a short while after each injection. Sternal recumbent position.	Lat.—poor. VD—indistinct.
4	60 lb. 4 yr. old	Air	80 cc. total given in 10–20 cc. aliquots; little reaction noticed. Sternal recumbent position.	Poor—air probably leaked out around needle.
3	35 lb. (old)	Air	6 cc. fluid withdrawn, 10 cc. air administered. Sternal recumbent position.	Poor—little contrast. TOTAL: poor, 3.
3	35 lb. (old)	Ethyl iodophenyl-undecylate	3 cc. given in lateral ventricle. 3 cc. given 1 hr. later and head rotated in various positions. Sternal recumbent position.	Good in laterals, third ventricle excellent, ventricles very large.
1	42 lb. 2 yr. old	Ethyl iodophenyl-undecylate	3 cc. followed by 5 cc. air. This was given immediately after failing with 50 cc. of air. Ethyl iodophenylundecylate seen 1 week later. Sternal recumbent position.	Fair—patchy filling of ventricles due to mixture of ethyl iodophenyl-undecylate and air.
2	58 lb.	Ethyl iodophenyl-undecylate	6 cc. in right lateral ventricle with needle in left. Sternal recumbent position.	Good—diffuse distribution. Much seen in cisterna. 6 cc. was too large a dose.
7	44 lb. 10 yr. old	Ethyl iodophenyl-undecylate	5 cc. in right side—right lateral recumbent position. Exposure made after each 1 cc. aliquot. DV radiographs made after nose held up and down for contrast migration. Contrast still present after 11 days.	Excellent outlining and contrast of all ventricles. Ventricles are large.
8	35 lb. 3 yr. old	Ethyl iodophenyl-undecylate	1.5 cc. given in each lateral ventricle. Sternal recumbent position.	Poor. Incomplete filling due to poor tap.
9	25 lb. 2 yr. old	Ethyl iodophenyl-undecylate	1 cc. injected in 0.5 cc. portions; moved head up and down between radiographs. Sternal recumbent position.	Poor. 3rd, 4th outlined. Mistaken tap into 3rd ventricle instead of lateral.
12	39 lb. 1 yr. old	Ethyl iodophenyl-undecylate	1 cc. given after tilting dog with head down, allowing fluid to drip out of needle. Sternal recumbent position.	Fair. Incomplete filling. Some contrast in subarachnoid.
13	33 lb. aged	Ethyl iodophenyl-undecylate given 40 days after pneumoencephalogram	3 cc. in left lateral ventricle in right lateral recumbent position.	Very good contrast and distribution. Ventricles seem same size as with air.
14	35 lb. 2 yr. old	Ethyl iodophenyl-undecylate	1 cc. in right side. Right lateral recumbent position.	Fair in laterals. 3rd-4th are good. Some contrast in subarachnoid.
61–902	Clinic case 9 yr. old	Ethyl iodophenyl-undecylate	3 cc. in right side. Right lateral recumbent position. Radiographs taken at 1st cc., 2nd cc., 3rd cc. injections.	Good filling of all ventricles—enlarged.
29	40 lb. 2 yr. old	Ethyl iodophenyl-undecylate	3 cc.—sternal recumbent position. Intermittent exposures in various positions. 30 cc. ringers injected into one side flowed from other.	Excellent for all ventricles. Very little medium removed.
30	70 lb. 10 yr. old	Ethyl iodophenyl-undecylate	3 cc.—exposures made after various positions. Given in left side, right lateral recumbent position.	Good distribution, good contrast.
34	35 lb. aged	Ethyl iodophenyl-undecylate	3 cc.—injected in right side. Left lateral position. 3 cc. more—no reaction seen.	All subarachnoid. Good distribution in ventricles.

TABLE 1 (cont.)—Results of Ventriculography by Using Several Contrast Mediums

Dog No.	Description	Contrast	Method	Results
35	18 lb. 7 mo. old	Ethyl iodophenyl-undecylate	1.5 cc. in right side. Left lateral position.	Good unilateral filling.
36	21 lb. aged	Ethyl iodophenyl-undecylate	2 cc. in right side. Left lateral recumbent position.	Fair filling.
37	25 lb. aged	Ethyl iodophenyl-undecylate	3 cc. in left side. Right lateral recumbent position.	Good filling—ventricles enlarged.
38	21 lb. 1 yr. old	Ethyl iodophenyl-undecylate	3 cc. in left side. Right lateral recumbent position.	Poor. All subarachnoid.
47	52 lb. 5 yr. old	Ethyl iodophenyl-undecylate	3 cc. in left side. Right lateral recumbent position.	Good unilateral filling.
				TOTAL: Good, 11 Fair, 4 Poor, 3
23	27 lb. 3 yr. old	Diatrizoate sodium	3 cc. in right side. Left lateral recumbency.	Good contrast and distribution. Died in 15 minutes in convulsions.
24	23 lb. 3 yr. old	Methylglucamine diatrizoate	6 cc. in left side. Sternal recumbent position.	Good contrast and distribution. Died in 1 hour.
27	25 lb. 2 yr. old	Methylglucamine diatrizoate	3 cc. in right side. Sternal recumbent position.	Poor contrast, fair distribution. Died 1½ hours later.
25	19 lb. 3 mo. old	Diatrizoate methylglucamine	3 cc. in right side. Right lateral recumbency.	Good contrast and distribution. Died 2 hours later.
26	33 lb. 3 yr. old	Sodium acetrizoate	3 cc. in right side. Sternal recumbent position.	Good contrast and distribution. Died in 5 minutes.
28	22 lb. 2 yr. old	N-propyl ester of 3:5 diiodo-4-pyridone-n-acetic acid	3 cc. in left side. Sternal recumbent position.	Fair distribution. Good contrast. No toxicity.
44	20 lb. 4 yr. old	Diluted to 50% with saline solution, n-propyl ester of 3:5 diiodo-4-pyridone-n-acetic acid	1 cc. in left side. Sternal recumbent position.	Good unilateral filling, good contrast. No reaction.
45	15 lb. 1 yr. old	Diluted to 33% with saline solution, n-propyl ester of 3:5 diiodo-4-pyridone-n-acetic acid	3 cc. in left side. Sternal recumbent position.	Contrast poor. Distribution good. No reaction.
49	50 lb. 3 yr. old	Diluted to 60% with saline solution, n-propyl ester of 3:5 diiodo-4-pyridone n-acetic acid	5 cc. in right side. Sternal recumbent position.	Fair contrast and good distribution. No toxicity seen.

Lat = lateral position; VD = Ventrodorsal position; DV = dorsoventral position.

and the needle was observed for a gentle rise of cerebrospinal fluid.

In some cases, the spinal fluid was withdrawn before air was introduced and in other cases it was not, but this did not seem to affect the results appreciably. In the average sized dog, 50 to 150 cc. of air was administered (Fig. 17). In some dogs there was obvious sensation as the air ascended in the cranial vault; in others there was not. The heads and bodies were positioned differently in an effort to obtain the optimum position for ventricular contrast. In most instances, the head was flexed at a 60-degree angle to that of the neck and, in other instances, the head was extended level with the neck and the table was in a nearly vertical position. This also did not seem to appreciably affect the ventricular contrast results.

Of these pneumoencephalograms, five lateral views were satisfactory and six were not satisfactory. In one instance, a satisfactory pneumoencephalogram was obtained but could not be duplicated in the same animal by introduction of air in the same manner (dog 5). Good dorsoventral radiographs were not obtained. The inconsistent results of introduction of air in this fashion seemed to bear out the findings in man; namely, that sometimes the air went into the ventricles and at other times it went into the cerebral subarachnoid space.[2] In some dogs, contrast was seen in both places—subarachnoid and intraventricular (Table 2).

Pneumoencephalography, Cisternal Route.—Pneumoencephalography via the cisterna magna route was also studied, since cisternal punctures were more easily

TABLE 2—Results of Pneumoencephalography Using the Lumbar Tap

Dog No.	Description	Contrast and amount	Method	Results
5	50 lb.	Air		
	3 yr. old	50 cc.	2–3 lumbar. Subarachnoid space—90°L tilt, head up and extended level.	Lateral—good; DV—fair.
		50 cc.	2–3 lumbar. Subarachnoid space—90°L tilt, head up and extended level.	Air stayed in ventricles 7 days.
	13 days later	100 cc.	1–2 lumbar. Table 75°L, head level.	Poor—no contrast.
		200 cc.	1–2 lumbar. Table 90°L, head level.	Poor—no contrast.
	40 days later	150 cc.	1–2 lumbar. Table vertical—head level.	Poor—very slight contrast.
10	27 lb.	Air		
	3 mo. old	50 cc.	1–2 lumbar. Table vertical—head level.	Poor.
		50 cc.	1–2 lumbar. Table vertical—head level.	Poor.
	50 days later	200 cc.	1–2 lumbar. Table vertical—head level.	Fair.
11	35 lb.	Air		
	6 mo. old	50 cc.	2–3 lumbar. Table vertical—head level.	Lateral—good; DV—fair.
		50 cc.	2–3 lumbar. Table vertical—head level.	Lateral—good; DV—fair.
13	33 lb.	Air	1–2 lumbar. Table vertical—head level.	Lateral—good;
	aged	50 cc.	Withdrew 10 cc. fluid.	DV—faint.
61-958	.....	Air		
		100 cc.	1–2 lumbar. Table vertical—head level.	Fair—lateral.
61-1228	60 lb.	Air		
	4 yr. old	150 cc.	1–2 lumbar. Table vertical—head level.	Good—lateral position. Fair—DV position.
40	26 lb.	Air		
	2 yr. old	50 cc.	1–2 lumbar. Table 75°L, head at 60°L to body.	Good—lateral position.
		50 cc.	1–2 lumbar. Table 85°L, neck held at 100°L, head at 90°L to neck.	Fair—DV position.
46	32 lb.	Air	1–2 lumbar. Table vertical—head level.	Poor.
	2 yr. old	100 cc.	1–2 lumbar. Table vertical—head at 45°L.	Poor.
				TOTAL— Good lateral, 5 Fair lateral, 2 Poor lateral, 4

Lateral or lat. = lateral position for radiograph; DV = dorsoventral position for radiograph.

TABLE 3—Results in Pneumoencephalography Using the Cisternal Tap

Dog No.	Description	Contrast and amount	Method	Results
6	55 lb. 2 yr. old	Air 50 cc.	Table tilted 45°L, head level. Withdrew 11 cc. fluid before air administration.	Poor—all subarachnoid.
		50 cc.	Table tilted vertical, head level.	Poor—all subarachnoid.
15	35 lb. 3 yr. old	Air 50 cc.	Table 45°L, head level. No fluid withdrawn at first—5 cc. fluid removed during administration of air.	Good on lateral, fair on DV.
		50 cc.	Same position—no fluid withdrawn.	Excellent on lateral, fair on DV
		50 cc.	Same.	No better.
18	50 lb. 7 yr. old	Air 150 cc.	Table 60°L, head held in 80°L.	Most in subarachnoid space—very little in ventricles.
		100 cc.	Table 60°L, head, 45°L.	Lateral good; DV, fair.
30	70 lb. 10 yr. old	Air 100 cc.	Table 85°L, head level.	Poor—all subarachnoid.
		100 cc.	Table 70°L, head level.	Poor—all subarachnoid.
43	35 lb. aged	Air 50 cc.	Table 75°L, head level.	Lateral, fair; DV, slight.
		50 cc.	Table 75°L, head 60° with neck.	Lateral, fair; DV, slight.
		75 cc.	Table 75°L, head level.	Lateral, fair; DV, slight.
61-1760	4 lb. 5 yr. old	Air 8 cc.	Table 80°L, head 60° with neck.	Lateral, good; DV, fair.
61-1680	8 lb. 3 yr. old	Air 30 cc.	Table 90°L, raise head 2" and head held at 90°L to neck.	Poor—subarachnoid filling.
50	60 lb. 3 yr. old	Air 100 cc.	Table 70°, head at 45°L to neck.	Poor—all subarachnoid.
				TOTAL—Good, 3 Fair, 1 Poor, 4

Lateral = lateral position for radiography; DV = dorsoventral position for radiography.

accomplished in the dog than lumbar punctures. For this route of administration, the animal was also anesthetized and the skin over the cisterna magna was prepared for aseptic puncture. The same type of spinal needle was introduced into the cisterna magna with the bevel of the needle directed anteriorly. After spinal fluid had been obtained in the usual fashion, the dog was tilted with the head up at a 70 to 80-degree angle in the sternal recumbent position (Fig. 18). Again, the position of the head was varied in order to find the position best adapted for this administration. In some dogs, air was administered with the head extended in the same plane as the cervical vertebrae; in others, the head was at a 45 to 60-degree angle to the cervical vertebrae. In others, the body was tilted to a 90-degree angle, the neck overextended to approximately a 100-degree angle, and the head at a 60-degree angle to the neck. Position again did not seem to affect the results appreciably.

Good results were obtained in 3 dogs, fair results in 1 dog, and poor results in 4 dogs. In the pneumoencephalograms that proved successful, the best ventricular contrast was seen in the straight lateral projection, whereas only faint contrast was usually seen in the dorsoventral position. This was probably true because of the petrous temporal bone interfering with radiographic visualization of the lateral ventricles (Fig. 19).

Besides air contrast via the lumbar and

cisterna subarachnoid routes, positive contrast mediums were also tried in 6 dogs: 1 with diatrizoate sodium; 4 with ethyl iodophenylundecylate; and 1 with n-propyl ester of 3:5 diiodo-4-pyridone-n-acetic acid. The dog given diatrizoate sodium died; in 1 dog given ethyl iodophenylundecylate there was very good contrast, whereas in 3 the material all went into the subarachnoid space; in the dog given n-propyl ester of 3:5 diiodo-4-pyridone-n-acetic acid, the medium also went into the subarachnoid space. In the one dog in which the materials seemed to flow into the ventricle, it was believed that the material was given in the central canal rather than in the subarachnoid space. It was determined that ventriculography with the positive medium, such as ethyl iodophenylundecylate, was far superior to such cisternal subarachnoid administration (Table 4).

case of diatrizoate sodium. Methylglucamine diatrizoate produced some small areas of hemorrhage adjacent to the fourth ventricle. When sodium acetrizoate was studied, numerous areas of hemorrhage were seen in the white substance of the cerebrum. There was also congestion of the meninges and some free hemorrhage in tissue adjacent to the lateral ventricles and an inflammatory response consisting of macrophage infiltration. When the four mediums were administered, hemorrhage seemed to be one of the main pathological lesions noticed. Three dogs given ethyl iodophenylundecylate the ventricles were studied histopathologically. In 1 of the 3, there were no significant lesions. In 1, a small accumulation of foreign material, undoubtedly ethyl iodophenylundecylate, was seen in the lining of one of the ventricles and was surrounded by macro-

TABLE 4—Results in Rhomboencephalography,* Using Several Contrast Mediums

Dog No.	Description	Contrast and amount	Method	Results
17	37 lb. aged	Diatrizoate sodium, 3 cc.	Dog tilted at 60°L with head down. Then tilted at 30°L and 80°L.	Poor—subarachnoid filling. Dog died in one hour.
19	35 lb. 2 yr. old	Ethyl iodophenyl-undecylate.	Tilted with head down 60°L for 10 minutes.	Excellent in 3rd and and 4th ventricles. Probably was administered in central canal.
20	26 lb. 3 yr. old	Ethyl iodophenyl-undecylate.	Tilted with head down for 15 minutes.	Poor—all subarchnoid.
21	35 lb. 3 yr. old	Ethyl iodophenyl-undecylate.	Tilted vertically, head down for 20 minutes.	Poor—all subarachnoid.
22	13 lb. 2 mo. old	Ethyl iodophenyl-undecylate, 3 cc.	Tilted vertically for 20 minutes.	Poor—all subarachnoid.
48	37 lb. 2 yr. old	N-propyl ester of 3:5 diiodo-4-pyridone-n-acetic acid diluted to 60% with saline solution, 6 cc.	Tilted 60°L, head down for 5 minutes—45 minutes.	Fair—ventricles appear very small.

* Injection of opaque contrast medium into the spinal subarachnoid space.

Toxicity Studies.—Toxicity studies were carried out on several dogs with the various agents used. It was mentioned that diatrizoate sodium, methylglucamine diatrizoate, sodium acetrizoate, and diatrizoate methylglucamine all produced toxic clinical results when these agents were introluced into the ventricles. Upon histopathologic study, large areas of hemorrhage adjacent to the ventricles were seen in the phages. In the third dog, there were small hemorrhages and folliculitis in tissues adjacent to the ventricles. Histopathologically, none of these lesions seemed to be severe.

N-propyl ester of 3:5 diiodo-4 pyridone-n-acetic acid administration was studied in 1 dog in which the administration had been by a direct ventricular tap and no significant changes were observed (Table 5).

TABLE 5—Toxic Responses of the Ventricular Linings to Various Contrast Mediums

Dog No.	Medium and site	Amount of medium	Interval between administration and necropsy	Lesions
7	Ethyl iodophenylundecylate; lat. vent.	5 cc.	12 days	Adjacent hemorrhage to lat. vent., some medium seen surrounded by macrophages. No inflammation in ventricular lining.
37	Ethyl iodophenylundecylate; lat. vent.	3 cc.	7 days	Slight inflammatory reaction in some lining cells. Cells contain clear substance in cytoplasm.
47	Ethyl iodophenylundecylate; lat. vent.	3 cc.	10 days	No significant lesions.
46	Air; 2–3L	200 cc.	10 days	Congestion of vessels in choroid plexus with small areas of hemorrhage.
13	Air; 1–2L	50 cc.	47 days	Slight increase in glial cells near ventricle in one section.
5	Air; 2-3L	500 c. on 3 occasions	40 days	No significant changes.
45	N-propyl ester of 3:5 diiodo-4-pyridone-n-acetic acid 33%	3 cc.	10 days	No significant changes.
24	Methylglucamine diatrizoate, 60%; lat. vent.	6 cc.	Died in 1 hour	Slight hemorrhage and gliosis in tissue adjacent to 4th ventricle.
26	Sodium acetrizoate, 60%; lat. vent.	3 cc.	Died in 5 minutes	Much hemorrhage in cerebral white substance. Congestion of dura.
25	Diatrizoate methylglucamine; lat. vent.	3 cc.	Died in 2 hours	Hemorrhage and necrosis adjacent to the lateral ventricle. Inflammatory response seen numerous places. Extensive hemorrhage in cerebral white.
23	Diatrizoate sodium; lat. vent.	3 cc.	Died in 1 hour	Hemorrhage adjacent to lateral ventricle.

Lat. vent. = lateral ventricle; 2–3L = 2nd-3rd lumbar interspace.

Tissue reactions to air administered to 3 dogs either by lumbar tap or through the cisterna magna were studied histopathologically. In 1 dog, there seemed to be a slight increase in the number of glial cells near the ventricle in one section. No other changes were noticed. In another dog, there was slight congestion of vessels in the chorioid plexus with small areas of hemorrhage, but no other significant changes were seen. In the third dog, given air either by lumbar or cisternal puncture on three different occasions, there were no significant changes (dog 5). Tissue changes were not seen at necropsy in response to any of the mediums administered, unless the animal involved had clinical signs.

Discussion

In these preliminary studies, it would seem that ventriculography is best accomplished with a positive medium such as ethyl iodophenylundecylate. Introduction of air into the ventricles seems to present many radiographic difficulties in the dog because of the heavy cranium, the petrous temporal bone, and the relatively small size of the ventricles. The density of the cranium in the dog seems to vary much more than that in man. However, the use of air in the spinal subarachnoid space, either at the cisterna magna level or at the lumbar level, seems to have some degree of promise. However, the results of relatively few such trials in this experiment were not consistent. It might be advisable to first try the lumbar air administration; if it fails, try the cisternal air administration; and, finally, use the ventricular administration of ethyl iodophenylundecylate.

The ventricular tap is relatively easy and seems to be safe. All of the procedures necessitate general anesthesia. Ventriculography requires an incision of the cranial skin and punctures made in the cranium for the entrance of the needle. Therefore, it must be considered the most drastic of the three procedures in that it involves actual surgery. Since no reaction was seen

from the administration of such contrast directly into the ventricles, it may even be safer than the pneumoencephalography. This has also been concluded by physicians after many years of experience.

It is possible that, with more investigation, air ventriculography might prove more successful in the dog than it would appear at the present time. Whenever air is administered for ventricular contrast, the amount must be exact. Since many veterinary practitioners do not have proper radiographic equipment and technical assistance available, it would seem that the radiopaque contrast ventriculography might be most practical.

References

[1] Davis, Loyal: *The Principles of Neurological Surgery.* 4th ed. Lea & Febiger, Philadelphia, (1953): 213.

[2] Davis, Loyal: *The Principles of Neurological Surgery.* 4th ed. Lea & Febiger, Philadelphia, (1953): 215–216.

[3] Fitzgerald, T. C.: Anatomy of Cerebral Ventricles of Domestic Animals. Vet. Med., 56, (1961), 38.

[4] Horwitz, H. H.: Positive Contrast Ventriculography —Critical Evaluation. J. Neurosurgery, 13, (July, 1956), 388.

[5] Mones, R., Werman, R.: Pantopaque Fourth Ventriculography via Lumbar Route. J. Mt. Sinai Hosp., New York, 25, (Mar.-Apr., 1958): 201–206.

[6] Orloff, T. L.: Danger from Thorotrast as Medium for Intracranial Angiography. Am. J. Surg., 95, (March, 1958), 469.

[7] Poppen, J. L.: An Atlas of Neurosurgical Techniques, W. B. Saunders, Philadelphia, (1960), 492.

[8] Robertson, E. G.: Pneumoencephalography. Charles C Thomas, Springfield, Ill., (1957): 71.

SUMMARIO IN INTERLINGUA

Encephalographia e Ventriculographia de Contrasto in le Can—Studios Preliminari

In studios preliminari, technicas esseva elaborate pro le execution de pneumoencephalographia in canes con le utilisation del via de administration cisternal o lumbar, sed le resultatos obtenite non esseva uniforme. Il pare que al tempore presente le injection de aere directemente ad in le ventriculos non es un practicabile methodo ventriculographic. Tamen, medios de contrasto radio-opac—como per exemplo iodophenylundecylato ethylic o estere n-propylic de acido 3:5-diiodo-4-pyridon-n-acetic—monstra grande promissas pro le ventriculographia canin.

The Chemical Analysis of Normal Canine Prostatic Fluid

Harris Rosenkrantz, Ph.D.; James Langille, B.A.; Marcus M. Mason, D.V.M.

SUMMARY

An extensive survey was made of the chemical constituents of canine prostatic fluid. Three noncastrated dogs without prostatic fistulas, 7 noncastrated dogs with prostatic fistulas, and 11 castrated dogs with fistulas were studied. Many of the findings of earlier investigators were confirmed but several new ovservations were made. It was found that the total nitrogen:dry weight ratio provided a relatively constant index for following prostatic activity. The total nitrogen could be categorized as approximately 50 to 60% protein, 20% polypeptide, 10% urea, and 10 to 15% other nonprotein nitrogen compounds, including catechol amines. The only reducing material of significant concentration (7%) was a nondialyzable polysaccharide or glycoprotein. The ash contributed about 25% to the total dry weight. The stability of prostatic acid phosphatase was also established.

INTRODUCTION

Although significant contributions to the chemical analysis of the prostatic secretion of the dog have been made,[7,9,10] few investigations have offered more than limited or fragmentary information. The recognition of the more simplified anatomy of the accessory organs of the reproductive tract of the dog highlighted the advantage of this species for studies in endocrinology.[2,5,8] With the development of surgical techniques for preparing dependable prostatic fistulas, careful chemical determinations of the composition of canine prostatic fluid could be undertaken.[10,12,13]

It has been clearly established that the pilocarpine-stimulated prostate of the dog elaborates significant quantities of organic and inorganic constituents.[7,10] The volume of the prostatic fluid varied from 7 to 30 ml. and the pH ranged from 6.1 to 6.8.[10] The concentration of osmotically active substances was 335 mEq./kg. of water and was comprised of 162.0 mEq. of sodium, 5.2 of potassium, 0.6 of calcium, 156.0 of chloride, 1.7 of bicarbonate, 0 to 3.0 of citrate, and a trace of phosphate.[7] The dry weight of 100 ml. of fluid was approximately 2.45 Gm.[10]

The organic constituents consisted of albumins, globulins, and nucleoproteins (866 mg./100 ml.); mucoproteins (57 mg./100 ml.); and lipids (40 to 180 mg./100 ml.). Several enzymes were detected: amylase, β-glucuronidase, fibrinogenase, traces of alkaline phosphatase, and relatively high concentrations of acid phosphatase (18 to 150 King-Armstrong units).[7,10] It has been demonstrated that the proteolytic activity of dog prostatic fluid was "tryptic-like" but was not inhibited by soybean trypsin inhibitor or by pancreatic trypsin inhibitor.[7]

Interestingly enough, despite the identification of fructose as the important energy substrate for sperm metabolism in other species, none or trace amounts of reducing sugars have been found in dog prostatic secretion. Some investigators have indicated the absence of sugars in the dog fluid [10] while others have detected the presence of glucose.[7]

The purpose of this investigation was to carry out an extensive quantitative analysis of the chemical constituents of canine prostatic secretion. Experiments were designed to account for all the dry weight present and to attempt to characterize the substances. Utilizing newer methods, simultaneous measurements on a relatively large sample of dogs was initiated. Both intact and castrated dogs with fistulas were stud-

Received for publication Dec. 19, 1960.

From the Department of Biology, Clark University, and the Biologics Testing Laboratory, Worcester, Mass.

This work was supported in great part by Cancer Chemotherapy National Service Center, National Cancer Institute, U.S. Public Health Service, Contracts SA-43-ph-1713 and SA-43-ph-2761.

The authors thank Drs. T. Boria and F. Keefe for the preparation and care of the animals; Esther Kirdani, John Perrone, Tom Barry, and Alex Fulop for competent technical assistance.

ied.[12] A few dogs were studied before any surgery.

Materials and Methods

The mixed-breed dogs under test weighed 11 to 42 kg. They were housed in individual plastic kennels and exercised in outdoor runs twice daily. Feeding was carried out twice daily, each dog's food intake being regulated according to condition, appetite, and weight gain or loss. The diet consisted of two parts commercial biscuit to one part canned beef. Powdered milk was prepared for the dogs twice weekly and multiple vitamin supplements were added to the daily ration.

The protatic fistulas were prepared according to published procedures.[13] For the collection of the prostatic secretion, the dogs were stationed on a table in a standing position and the fluid was collected into graduated centrifuge tubes for one hour. Earlier experiments employed a standard i.v. dose of 5 mg. of pilocarpine, whereas more recent studies used doses related to body weight (0.7 mg./kg.). Prostatic fluid was collected two to three times per week for each animal.

All castrated and vasectomized dogs were given 10 mg. of testosterone subcutaneously every day.

Where the volume of prostatic fluid was not limiting, duplicate determinations of dry weight, ash, sodium, potassium, lipids, total nitrogen, nonprotein nitrogen, creatinine, creatine, urea, ammonia, and reducing sugars were performed. Polymer determinations of protein and polysaccharide were carried out after dialysis. Acid phosphatase was also estimated and pH was measured electronically. Insoluble material was removed by centrifugation before any chemical analyses were made.

The dry weight was obtained on a 0.3- to 0.5-ml. aliquot of prostatic secretion in a tared 5.0-ml. beaker heated until constant weight was reached. All samples were cooled to room temperature in a desiccator before they were weighed. After obtaining the dry weights, the residue was redissolved in water and extracted three times with 10 ml. of ether for the estimation of lipids on a weight basis. Another 0.5-ml. aliquot of the prostatic fluid was ashed in a porcelain crucible over a Bunsen burner to constant weight. The residue was redissolved in water and sodium, and potassium was measured by flame photometry.[6]

For an accounting of nitrogen-containing constituents, total nitrogen was determined on a 0.5-ml. aliquot of original fluid by the usual Kjeldahl-nesslerization methods. Nonprotein nitrogen (NPN) supernatant fluids were prepared by precipitation of the proteins with $ZnSO_4$ and $Ba(OH)_2$ (1 ml. prostatic fluid to 2.0 ml. of 4.7% $ZnSO_4$ and 2.0 ml. of 0.3N $Ba[OH]_2$). The supernatant fluids were utilized for the estimation of NPN by the Kjeldahl-nesslerization methods,[6] creatinine by the alkaline picric acid procedure,[6] creatine by the diacetyl method,[8] and urea via the urease technique.[6] Specific reducing sugars were estimated as follows: fructose by the resorcinol method,[21] glucose via arsenomolybdic acid,[1] and ribose by the orcinol method.[1]

Polysaccharide was determined by a modification of the naphthol ring test.* An aliquot of 0.1 ml. of original prostatic fluid was diluted with 1.9 ml. of distilled water and put in an ice bath. One drop of 15% *beta* naphthol in 95% ethanol was added and dispersed. The solution was underlayed with 5.0 ml. of concentrated sulfuric acid and the tubes were transferred to a boiling water bath for five minutes, shaken thoroughly, cooled to room temperature, and read at 574 mμ.

High molecular weight substances were isolated by dialysis; 2 to 3 ml. of prostatic secretion was placed in Visking tubing 3 cm. in diameter and dialyzed against 1 liter of distilled water for 24 hours. The distilled water was changed three times during this period. Protein nitrogen, polysaccharide, and dry weight was measured as mentioned previously.

The opportunity was available for determining acid phosphatase, using a glycerophosphate substrate-buffer [16] and catechol amines by fluorometry.[15] Original prostatic fluid (0.1 ml.) was used for a study on the levels of phosphatases. In addition to the estimation of enzyme activity from day to day, some samples were analyzed each day for a week to evaluate the stability of the enzyme and prostatic fluid. The samples were kept at 5 C., when aliquots were not being analyzed.

Results

The dogs under investigation have been studied for several months and the ranges of the parameters have been fairly well established. Some of the history of each animal and the range of volumes collected in one hour are listed (Table 1). The volumes represent the response of the prostate gland to a single i.v. injection of 5 mg. of pilocarpine hydrochloride. The range of volumes represents at least six determinations on each dog. In the case of dogs 424, 425, and 428, the prostatic fluid was obtained by masturbation and fractional collection; *i.e.*, the first fraction was collected in four minutes, and the second fraction was collected during an additional 15 minutes. Centrifugation at 3,000 r.p.m. for half an hour and microscopic examinations assured that sperm was absent in this latter fraction. The sperm in the early fraction could also be removed by centrifugation and the fluid analyzed for chemical constituents.

No clear-cut correlation existed (Table

* Rosenkrantz, H., Forchielli, E., Giering, J., and Truant, A. P.: Unpublished method developed in December, 1958, at the Astra Pharmaceutical Company, Worcester, Mass.

TABLE 1—The Volume and pH of Canine Prostatic Secretion*

Dog (No.)	Breed	Age (yr.)	Body weight (kg.)	Treatment	Volume (ml.)	pH
353	Collie x	7	21.8	Noncastrated	6.0–9.0	6.7–7.2
373	Hound x	2	16.3	Noncastrated	0.5–1.0	.....
374	Hound x	2½	18.2	Noncastrated	2.0–6.0	7.0–7.2
385	Beagle	1	11.4	Noncastrated	3.0–10.0	6.8–7.2
386	Collie x	3	18.2	Noncastrated	1.0–5.0	6.4–7.1
387	Shepherd	2	29.6	Noncastrated	0.5–3.0	6.6–6.8
362	Beagle	8½	14.5	Castrated	1.0–3.0	6.8–7.1
366	Hound x	1½	15.0	Castrated	0.5–2.0	6.8–7.2
367	Toy Shepherd	1½	15.0	Castrated	1.0–2.0	6.6–6.8
368	Beagle x	2	7.7	Castrated	8.0–14.0	5.9–6.7
369	Beagle	3½	16.8	Castrated	4.0–9.0	6.5–6.9
375	Lab. Ret. x	1½	16.8	Castrated	1.0–3.0	6.5–6.9
379	Setter x	2	15.0	Castrated	2.0–7.0	6.9–7.1
381	Foxhound x	2	21.8	Castrated	1.0–7.0	.....
424	Collie x	6	21.4	No surgery	29.0–35.0**	6.7–7.3
425	Setter x	4	24.5	No surgery	5.0–14.0**	.....
428	Collie x	1½	20.0	No surgery	8.0–11.0**	.....

* All dogs had direct fistulas except: 385 and 381 (cystostomy); 386 (cystopreputiostomy); and 379 (cystoileostomy).

** All dogs were given one i.v. injection of 5 mg. of pilocarpine hydrochloride except 424, 425, and 428. The latter were stimulated by digital manipulation.

1) between the volume of prostatic fluid and age or weight of the animals. In the noncastrated dogs, the heaviest (387) elaborated less secretion than the lightest (385). However, the oldest (353) secreted more fluid than the younger animals. In the castrated dogs, 368, not the youngest but certainly the lightest dog, produced the largest volumes.

In most instances, pH measurements were between slightly acid to slightly basic.

It was obvious from the variable output of volumes that a suitable expression of the levels of chemical constituents would be needed. It did not seem reasonable to express values in mg./100 ml. of prostatic fluid (as in the literature) since it would mean multiplying any errors by a large factor. Since the volumes in reality were low, it was decided to record titers in mg./ml. This would tend to be an index of the richness of fluid collected under identical circumstances and be independent of total volume. Therefore, in subsequent tables the results are expressed in this manner.

The concentrations of inorganic materials are listed (Table 2). The information in this table reveals the significant content of dry weight material. The over-all range for noncastrated dogs was between 16.1 to 25.9 mg./ml., while the values were between 16.7 to 37.3 for castrated dogs given testosterone. Except for dog 379, there appeared to be no question that animals given

TABLE 2—Inorganic Constituents of Canine Prostatic Fluid*

Dog (No.)	Dry weight (mg./ml.)	Ash (mg./ml.)	Sodium (mg./ml.)	Potassium (mg./ml.)
NONCASTRATED				
353	19.6–20.9	6.9–7.5	1.21–1.77	0.42–0.62
374**	22.2–25.9	2.1–2.7	0.43–0.53	0.14–0.22
385	17.7–22.5	5.5–7.1	1.01–1.85	0.31–0.48
386**	16.6–18.1	.....	.....	.....
387**	16.9–23.7	5.7–6.8	1.08–1.68	0.33–0.53
424	16.1–17.8	.....	.....	.....
425	18.2–20.4	.....	.....	.....
CASTRATED				
362	22.1–29.0	8.5–10.4	0.75–0.95	0.30–0.50
366	29.1–36.0	.....	.....	.....
367	24.9–33.8	4.8–6.3	0.98–1.16	0.43–0.62
368	24.7–29.8	4.2–5.2	1.09–2.26	0.33–0.58
369	25.5–29.4	5.4–6.1	0.69–1.36	0.29–0.35
375	23.3–37.3	.....	.....	.....
374	21.9–23.3	.....	.....	.....
386	21.4–27.6	.....	.....	.....
387	20.5–28.7	8.1–9.5	2.05–2.45	0.81–0.93
379	16.7–20.8	2.5–2.8	0.42–0.62	0.14–0.24
381	25.9–33.1	.....	.....	.....

* Three to six determinations made for each parameter. ** These dogs were studied before and after castration; dog 374 was given supplements of testosterone before and after castration.

testosterone supplement produced a fluid richer in solids. This suggested that the levels of exogenous hormone were higher than the endogenous levels manufactured and required by the animals themselves.

The data in Table 2 also disclose that the inorganic constituents (ash) comprised, generally speaking, between 15 to 35% of the total dry weight. Only sodium and potassium were determined in this investigation, and it was found that approximately 20 to 30% of the ash was sodium and 5 to 10% was potassium. Other investigators have demonstrated that chloride occurs in concentrations similar to sodium.[7] In the present study, trace or no levels of inorganic phosphate could be detected. An accounting of the ash indicates that approximately 30% of other inorganic constituents must be sought.

From the previous discussion, it can be seen that the major component of the solids in dog prostatic fluid must be organic material. The analysis for the usual classifications of organic matter as protein, carbohydrates, and lipids is given (Table 3).

weight levels, protein titers appeared higher in testosterone-treated, castrated dogs than in intact dogs.

The NPN accounted for approximately 20% of the total nitrogen. Urea was responsible for approximately 50% of the NPN, whereas ammonia contributed 6%. Negative results were obtained for creatinine and creatine.

The concentration of carbohydrate varied from 0.65 to 5.0 mg./ml. (approx. 7% of the dry weight) and this large variation may be in part a reflection of the presence of an amylase.[10] It was observed that carbohydrate titers could decline in aged samples. When specific tests for monosaccharides were utilized, it was found that a few samples of prostatic fluid contained traces (less than 0.05 mg./ml.) of glucose and ribose. It is to be emphasized that in most instances negative results were obtained for fructose as well as glucose and ribose.

The levels of lipids were uniformly low and accounted for approximately 2.4% of the total dry weight. A total accounting then of the average dry weight sample was

TABLE 3—Organic Constituents of Canine Prostatic Fluid*

Dog (No.)	Total nitrogen (mg./ml.)	Nonprotein nitrogen (mg./ml.)	Carbohydrate (mg./ml.)	Lipids (mg./ml.)
NONCASTRATED				
353	1.68–1.92	0.44–0.85	0.70–2.95	0.26–0.47
374**	2.09–2.89	0.27–0.45	1.23–1.86	0.17–0.22
385	1.64–1.96	0.35–0.67	0.79–2.10	0.60–0.96
386**	1.60–1.98	.....	.....	0.95–1.42
387**	1.81–2.37	.....	.....	0.90–1.18
424	1.15–1.30	.....	.....	.....
425	1.38–1.84	.....	.....	.....
CASTRATED				
362	3.02–3.20	0.42–0.50	1.25–1.92	0.20–0.40
366	3.65–4.58	.....	.....	.....
367	2.95–4.29	0.35–0.45	2.20–2.40	0.31–0.39
368	2.94–3.52	0.28–0.47	0.95–5.00	0.35–0.45
369	2.68–3.28	0.49–0.85	1.15–1.32	0.33–0.37
375	2.58–4.35	.....	0.80–1.80	0.27–0.37
374	2.26–2.46	0.23–0.30	.....	.....
386	2.61–3.01	.....	.....	.....
387	1.62–3.08	.....	.....	.....
379	1.14–2.04	0.33–0.49	0.65–0.85	.....
381	2.62–3.37	.....	1.91–3.01	0.30–0.42

* Three to six determinations were made for each parameter. ** These animals were studied before and after castration; dog 374 was given supplements of testosterone before and after castration.

It can be seen from the data in columns 2 and 3 that canine prostatic secretion is relatively rich in protein material. Employing the difference between the values in these two columns and the dry weights given in Table 2, it was found that the range of protein substances for all dogs was approximately 40 to 65%. As in the case of dry

approximately as follows: ash, 25.0%; lipids, 2.4%; carbohydrate, 6.9%; protein, 56.0%; and NPN, calculated mainly as urea, 4.7%. This totals 95% of the solids.

It was of considerable interest to further characterize the nitrogen and carbohydrate constituents. Several dogs on hormone treatment were selected for analysis of their

prostatic secretions before and after dialysis. The results of these experiments are shown (Table 4). The nondialyzable nitrogen calculated as true protein was equivalent to approximately 38% of the total dry weight and 62% of the total nitrogen before dialysis. Since nonprotein nitrogen estimations before dialysis accounted for about 20% of the total nitrogen, then approximately 20% of the total nitrogen would appear to be polypeptide in nature (molecular weight less than 1,000). As would be expected, the polypeptides would be precipitable by the zinc sulfate-barium hydroxide system and pass the dialyzing membrane.

TABLE 4—Partial Characterization of Canine Prostatic Fluid Components by Dialysis

Dogs with fistulas		mg./ml. before dialysis			mg./ml. after dialysis		
	Treatment*	Dry wt.	Total N	CHO**	Dry wt.	Total N	CHO**
353, intact	None	20.6	1.83	0.45	6.7	1.12	0.49
	Stilbestrol	18.0	1.62	0.25	9.4	1.38	0.25
374, intact	Test. + stilb.	31.6	3.44	0.50	10.5	1.31	0.47
368, castrated	Testosterone	24.7	2.46	1.66	9.7	1.35	1.60
	Test. + stilb.	29.9	3.32	0.65	10.2	1.45	0.64
	Testosterone	27.4	2.95	0.90	16.0	2.60	0.95
369, castrated	Testosterone	33.1	3.54	0.50	13.1	1.93	0.50
	Test. + stilb.	33.4	3.65	.....	17.0	2.88	.....
375, castrated	Test. + stilb.	22.5	2.15	0.70	12.7	1.75	0.90
379, vasectomy (cystoileostomy)	Stilbestrol	16.1	1.13	0.29	3.1	0.36	0.30

* Stilbestrol was given at a rate of 1 mg. daily; testosterone was given in 10-mg. doses. ** Total carbohydrate.

During the precipitation of proteins for the preparation of NPN supernatant fluids, it was observed that the carbohydrate material would co-precipitate. This phenomenon was suggestive of a polysaccharide or glycoprotein. The data in Table 4 further substantiate the molecular size of the carbohydrate since it was nondialyzable. The sum of the weights of the nondialyzable protein and carbohydrate satisfactorily accounted for the total dry weight residing within the dialyzing bag. It may be generally stated that stilbestrol treatment tended to decrease carbohydrate titers.

Other explorations were undertaken for nitrogen-containing constituents. A fluorescent procedure for catechol amines was applied to a number of samples of prostatic fluid (Table 5). Addition of 0.05 or 0.10 μg. of norepinephrine and epinephrine, respectively, to 1 ml. of prostatic fluid could be recovered between 90 to 100% without fractionation of the fluid. This suggested that little or no interfering compounds were present. The results on the determination of the catechol amines are outlined (Table 5). A range of 0.07 to 0.60 μg./ml. was obtained for epinephrine and values for norepinephrine varied similarly. In most instances, titers of epinephrine were higher than the demethylated analogue.

Since the determination of total nitrogen and dry weight for canine prostatic secretion appeared to be a measure of the richness of the fluid, a ratio of both parameters was used for comparison with other species. This total nitrogen : dry weight ratio was quite constant from day to day

TABLE 5—Catechol Amine Content of Canine Prostatic Fluid*

Dog (No.)	Date sample collected	Norepinephrine μg./ml.	Epinephrine μg./ml.
353	7–28	0.40	0.24
366	8–4	0.60	0.30
367	8–4	0.50	0.50
368	8–2	0.50	0.50
368	10–14	0.03	0.14
369	8–3	0.25	0.24
369	10–10	0.40	0.14
369	10–17	0.05	0.09
374	10–3	0.05	0.15
374	10–6	0.07	0.07
374	10–13	0.23	0.17
374	10–17	0.08	0.14
379	9–29	0.07	0.42
379	10–10	0.14	0.23
381	10–4	0.09	0.08
381	10–11	0.13	0.17
385	7–26	0.50	0.60
387	10–4	0.06	0.25
387	10–7	0.23	0.35
387	10–11	0.10	0.15
387	10–14	0.23	0.41

* Dogs 353 and 385 had no treatment; all others were given testosterone. Blood values for the dog have been reported as being 0.61 ± 0.50 s.d. and 1.25 ± 0.86 mμg./min./kg. for norepinephrine and epinephrine, respectively.[4] Levels of 0.18 mg./Gm. and 0.87 mg./Gm. of norepinephrine and epinephrine, respectively, for the adrenals of dogs have been found.[14]

TABLE 6—Dry Weight and Total Nitrogen Determination on Seminal Plasma of Various Species*

Animal	mg./ml. Dry wt.	Total N	Protein	Protein (%)	Total N / Dry wt.
Man	82.0	9.13	52.3	63	0.111
Bull	95.3	7.56	44.2	46	0.080
	94.9	8.0	45.2	48	0.084
Ram	148.2	8.75	51.1	34	0.059
Boar	172.3	13.96	85.0	50	0.083
Stallion	24.5	1.67	7.0	29	0.069
Dog**					
Intact	19.9	1.95	10.4	52	0.098
Castrated	29.6	3.28	16.4	55	0.111

* Data taken from reference.[10] ** The values for the dog are the mean of 4 noncastrated (intact) and 10 testosterone-supplemented castrated dogs. All had prostatic fistulas.

for each dog. The data in Table 6 depict the pertinent comparison among the species. Values for the dog more closely approximate those of man than do those of the other species. The constancy of the total nitrogen : dry weight ratio suggested utilization of this index in following the period of time that the dogs would require to recover from surgical preparation. This information has been recorded in Table 7.

the testosterone-treated castrated dogs tended to elaborate higher titers of acid phosphatase. The average mean for intact dogs was 7.3; for castrated dogs the value was close to 10.9 mg. of PO_4 released per hour per milliliter of prostatic fluid.

TABLE 8—The Levels of Acid Phosphatase in Canine Prostatic Fluid

Dog. No. and condition	Av. vol. (ml.)	Total No. determinations	mg. PO_4 released/hr./ml. Range	Mean ± a.d.
353, intact	6.7	6	4.3–7.0	6.1 ± 0.9
374, intact	1.6	4	4.4–5.8	5.1 ± 0.4
385, intact	8.0	4	5.5–7.2	6.3 ± 0.7
386, intact	4.0	3	7.9–9.2	8.6 ± 0.4
387, intact	2.1	5	9.5–11.9	10.6 ± 0.8
362, castrated	3.0	5	6.0–10.0	8.3 ± 1.3
369, castrated	5.0	7	4.8–8.8	6.6 ± 1.0
367, castrated	4.3	2	12.5–13.1	12.8 ± 0.3
368, castrated	15.7	4	12.4–17.6	15.0 ± 1.5
379, castrated	6.4	5	6.7–9.5	8.4 ± 0.9
381, castrated	6.7	6	10.9–19.3	14.5 ± 2.0

The procedure for the estimation of acid phosphatase was also used to follow the stability of this enzyme in the prostatic fluid. It was found (Table 9) that enzyme titers remained constant for at least seven days within the experimental error of the method. Microscopic examination of each fluid each day failed to reveal the presence of bacteria. The pH did not significantly change during the one-week period of study.

TABLE 7—Total Nitrogen: Dry Weight Levels in Prostatic Fluid of Dogs Just After Surgery

Dog (No.)	Fistula approach	Weekly determinations mg. nitrogen/ml. : mg. dry weight/ml. 1	2	3	4	5	6	7	8	9	10
387	Direct	0.096	0.109	0.102	.....	.....	0.100	0.098	.....	.....	0.098
386	Cystopreputiostomy	0.187	0.162	0.085	0.089	.....	.....	0.100	0.093	0.101	0.102
385	Cystostomy	0.171	0.137	0.145	0.093	0.094	0.110	0.090	.....	0.091	0.102
381	Cystostomy	0.186	0.197	0.134	0.142	.....	0.110	0.114	0.101	.....	0.092

Each dog was studied after a two-week period necessary for convalescence. The total nitrogen : dry weight ratio became relatively constant almost immediately after convalescence, in the case of the direct fistula approach. The urethral adaptations caused elevations in total nitrogen, but the ratio stabilized three to five weeks after convalescence.

One of the more extensively studied enzymes in canine prostatic fluid has been acid phosphatase.[7] Whereas previous investigators employed a synthetic substrate, phenyl phosphate, this study incorporated a natural substrate, glycerophosphate.[16] The variability in the titer of this enzyme during weekly determinations is shown (Table 8). There was no apparent correlation between the levels of acid phosphatase and the age or weight of the animal. However,

During the present investigation, chemical measurements were carried out on dogs before they underwent surgery for fistula construction. The seminal plasma was obtained by manual manipulation, and the early sperm-containing fraction was collected separately from the subsequent fluid mainly composed of prostatic secretion. Both fractions were analyzed (Table 10) after removal of sperm and insoluble debris by centrifugation. The information in Table 10 clearly reveals that the total nitro-

TABLE 9—Stability of Acid Phosphatase in Canine Prostatic Fluid*

Dog (No.)	No. of determinations on same samples on consecutive days	mg. PO_4/hr./ml. fluid	
		Range	Mean ± a.d.**
368	5	11.4–12.4	11.9 ± 0.3
369	6	6.3– 8.2	7.1 ± 0.5
385	5	4.3– 5.8	5.0 ± 0.5
368	7	12.3–14.5	13.3 ± 0.8
381	7	12.4–15.2	13.5 ± 0.8
386	7	7.9– 9.1	8.4 ± 0.4
387	7	10.3–12.1	11.1 ± 0.5
353	6	4.9– 5.7	5.3 ± 0.4
362	6	8.3– 9.4	8.8 ± 0.3
379	6	9.5–10.3	9.9 ± 0.3
381	6	12.9–14.6	13.8 ± 0.4
386	6	7.9– 8.6	8.3 ± 0.1
387	6	6.7– 7.3	7.0 ± 0.2

* Duplicate determinations were made on the samples on their day of arrival and thenceforth on the number of consecutive days indicated. The fluids were kept at 5 C. between analyses. There was no significant variation in pH during the study and the instance of bacterial detection microscopically was rare.

** The over-all percentage error for all determinations was approximately 5, range 1 to 10%.

gen : dry weight ratio is in the range observed for dogs with fistulas. The total nitrogen and dry weight values were about doubled in the 15-minute fraction in dog 424 as compared with those dogs with fistulas, but this was the first attempt at collecting prostatic fluid by masturbation. The corresponding values for dogs 425 and 428 were remarkably similar to those of dogs with fistulas. Secretions from organs other than the prostate contributed to the higher concentrations of material in the two- to four-minute fraction, as would be expected.

In more recent explorations, because fluid volumes were low, attempts at elevating the volume of secretion were undertaken. Six intact and 6 castrated dogs were given 0.7 mg./kg. of pilocarpine for stimulation of the prostate gland. In all instances, an increase in the volume of prostatic fluid resulted. The increment was between 2.5 to 7.0 times the original volume obtained at the pilocarpine dose of 5.0 mg. per dog. Similar to the use of one hour for collecting fluid in the earlier experiments, it was found that at least one hour was necessary to collect the prostatic secretion released by the higher dose of pilocarpine (Fig. 1). As can be seen in Figure 1, volume plateaus were being reached at about one hour for most of the animals studied.

Discussion

The endeavors of Huggins and colleagues over the past 20 years have illuminated many avenues of exploration on the prostate gland and its physiologically important secretion.[7] Additional information is sorely needed for elucidating the mechanisms of synthesis of the gland, the purpose of the products released, and the integrated influences of hormones on the gland. The anatomic advantage of the dog should stimulate further studies in these directions.

The approach in the present investigation has been to make an accounting of the chemical constituents in prostatic fluid. It was also hoped that a battery of dependable parameters could be evolved to follow the activity of the prostate gland. The data presented show that the concentration of protein material could be an important

TABLE 10—Chemical Analyses on Canine Seminal Plasma*

Dog (No.)	Date	Collection time (min.)	Volume (ml.)	Dry weight (mg./ml.)	Total nitrogen (mg./ml.)	Total N / Dry wt.
424	7–28	2.0	5.5	82.7	7.9	0.095
		15.0	41.5	49.8	4.3	0.086
424	8–1	2.0	9.2	71.3	7.2	0.101
		11.0	28.6	43.5	3.5	0.081
424	8–4	2.0	6.9	68.7	8.2	0.119
		20.0	26.0	44.8	4.2	0.094
425	8–1	3.0	6.3	67.9	6.8	0.100
		8.0	10.0	21.0	2.1	0.100
425	8–5	2.5	3.0	65.4	6.9	0.105
		10.0	5.2	24.8	2.6	0.105
425	8–8	4.0	6.4	37.5	3.9	0.104
		15.0	5.8	22.9	2.4	0.104
428	9–28	4.0	5.5	44.6	4.9	0.110
		12.0	7.7	21.5	1.8	0.084
428	10–1	4.0	5.0	44.3	4.8	0.108
		14.0	10.8	23.5	2.1	0.089
428	10–5	5.0	4.8	40.9	4.5	0.110
		18.0	10.8	21.3	1.9	0.089

* Approximately 25% of total nitrogen was nonprotein nitrogen. Carbohydrate amounted to 7%.

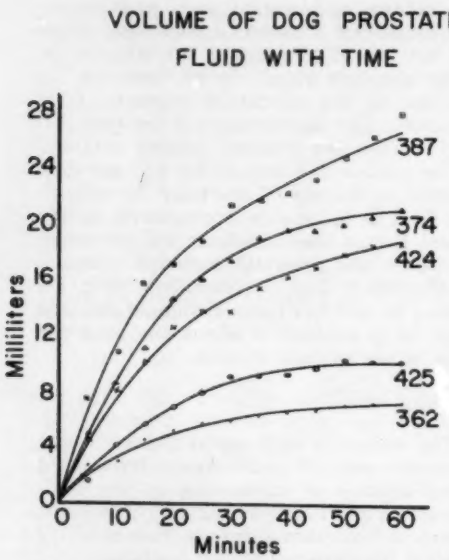


Fig. 1—Graph showing the volume of prostatic secretion during a one-hour collection period for several dogs with prostatic fistulas. (The numbers within the graph designate individual dogs.)

parameter for evaluating prostatic activity. It was found that the relatively high concentration of protein nitrogen was indicative of the richness of the fluid. It was obvious that castration reduced the protein (and enzyme) content and that administration of testosterone resulted in an increment. This would certainly suggest a direct hormonal response.

Other interesting nitrogen components that require elucidation are the dialyzable constituents that may be polypeptides. It is not unreasonable to suppose that these compounds may play an important physiologic role.

A similar situation may exist for the polysaccharide or glycoprotein found to occur in significant quantities. Here is the first indication of a possible source of energy for dog sperm. The absence of significant concentrations of fructose or glucose in canine prostatic fluid, unlike findings in many other species, is curious. The possibility that the reducing polymer may also be useful in hormone bioassay was suggested by the decrease in its concentration when the dogs were treated with stilbestrol.

Another interesting observation made was the finding of both norepinephrine and epinephrine in prostatic fluid. Here again an explanation must be found for the presence of these hormones in the natural environment of sperm. Speculation may range from an activation of phosphorylase activity in sperm to an influence on the contractility of the female genital tract.

Although approximately 95% of the total solids have been categorized, much work remains to be carried out for the characterization of each component. The possibility of analyzing prostatic fluid on intact dogs through fractional collection is not being overlooked. The similarity of protein concentrations between canine and human prostatic secretion suggests that information derived from the dog may be applicable to man.

References

[1] Ashwell, G.: Arsenomolybdate Method of Sugar. In Colowick and Kaplan's *Methods in Enzymology. III.* Academic Press, New York, (1957): 85–86.

[2] Berg, O. A.: Effect of Stilbestrol on the Prostate Gland in Normal Puppies and Adult Dogs. Acta Endocrinol., 27, (1958): 140–169.

[3] Enner, A. H., and Stocken, L. A.: Application of the Diacetyl Reaction to the Estimation of Creatine in Urine. Biochem. J., 55, (1953): 310–314.

[4] Goldfien, A., and Ganong, W. F.: Stimulation of Various Parts of Dog Hypothalmus to Release Catechol Amines. Fed. Proc., 17, (1958): 56.

[5] Harrop, A. E.: Some Observations on Canine Semen. Vet. Rec., 67, (1955): 494–498.

[6] Hawk, P. B., Oser, B. L., and Summerson, W. H.: *Practical Physiological Chemistry.* 13th ed. McGraw-Hill, New York (1954): 548–650.

[7] Huggins, C.: The Prostatic Secretion. Harvey Lect., J. B. Lippincott & Co., Philadelphia, (1946–47): 148–193.

[8] Huggins, C., Masina, M. H., Eichelberger, L., and Wharton, J. D.: Quantitative Studies of Prostatic Secretion. Characteristics of the Normal Secretion, the Influence of Thyroid, Suprarenal, and Testis Extirpation and Androgen Substitution of the Prostatic Output. J. Exptl. Med., 70, (1939): 543–556.

[9] Huggins, C., and Sommer, J. L.: Quantitative Studies of Prostatic Secretion. III. Simultaneous Measurement of Size and Secretion of the Canine Prostate and the Interaction of Androgens and Estrogenic Substances Thereon. J. Exptl. Med., 97, (1953): 663–674.

[10] Mann, T.: *The Biochemistry of Semen.* Methuen & Co., London, England, 1954.

[11] Mann, T.: Fructose Content and Fructolysis in Semen. Practical Application in Evaluation of Semen Quality. J. Agric. Sci., 38, (1948): 323–333.

[12] Marden, H. E., Jr., Grayhack, J. T., and Scott, W. W.: A New Prostatic Fistula. J. Urol., 73, (1955): 703–708.

[13] Mason, Marcus M., Keefe, Fred, and Boria, Thomas: Specialized Surgery of the Canine Bladder and Prostate Gland—The Isolation and Collection of Prostatic Fluid. J.A.V.M.A., 139, (Nov. 1, 1961): 1007–1015.

[14] Ozaki, T.: Noradrenaline and Adrenaline Contents of Suprarenal Glands in Various Species of Animals. Tohoku J. Exptl. Med., 61, (1955): 345–352.

[15] Roston, S.: Fluorometric Determination of Adrenaline and Noradrenaline in Aqueous Solution. Anal. Chem., 30, (1958): 1360–1366.

[16] Shinowara, G. Y., Jones, L. M., and Reinhart, H. L.: The Estimation of Serum Inorganic Phosphate and "Acid" and "Alkaline" Phosphatase Activity. J. Biol. Chem., 142, (1942): 921–933.

SUMMARIO IN INTERLINGUA

Le Analyse Chimic de Normal Liquido Prostatic Canin

Esseva facite un extense studio del constituentes chimic de canin liquido prostatic. Esseva studiate 3 non-castrate canes sin fistulas prostatic, 7 non-castrate canes con fistulas prostatic, e 11 castrate canes con fistulas prostatic. Multes del constatationes de previe investigatores esseva confirmate, sed plure nove observationes esseva facite. Esseva trovate que le proportion nitrogeno total a peso sic provideva un relativemente constante indice pro observar le activitate prostatic. Le nitrogeno total poteva esser categorisate como continite a circa 50 a 60% in proteina, a 20% in polypeptido, a 10% in urea, e a inter 10 e 15% in altere compositos a nitrogeno non-proteinic, incluse catecholaminas. Le sol material reducente de grados significative de concentration (7%) esseva un non-dialysabile polysaccharido o glycoproteina. Le cineres contribueva circa 25% al total peso sic. Le stabilitate del prostatic phosphatase acide esseva etiam demonstrate.

Further Studies on Prophylactic Therapy Against the Developing Stages of *Dirofilaria immitis* Before Reaching the Canine Heart

Seiji Kume, D.V.M., Ph.D.; Isamu Ohishi, D.V.M., Ph.D.; Shigeo Kobayashi, D.V.M.

SUMMARY

Diethylcarbamazine, an antimonial (sodium antimony-III-biscatechol disulfonate), and an arsenical (dichlorophenarsine hydrochloride) were tested for their ability to destroy *Dirofilaria immitis* worms in their intermediate developmental location before they reach the heart. As prophylactic agents, none of the compounds was completely satisfactory or practical in the dosages tested.

INTRODUCTION

When Kume [3] reported success in preventing the development of *Dirofilaria immitis* in experimentally infected dogs with antimony at twice the microfilaricidal dose, it was the first concrete evidence that any therapeutic agent was prophylactic. It was suggested that the minimum protective dose of antimonials be determined more accurately and that it be determined whether antimonials may be utilized as practical prophylactic agents.

Field trials [1] have indicated that diethylcarbamazine* can prevent *Dirofilaria* infection. A dosage of 25 mg. of diethylcarbamazine per pound of body weight daily for three days has been suggested as the preventive dosage. However, it was not known whether diethylcarbamazine destroyed the young worms in the intermediate developmental location (submuscular membranes, subcutaneous fat, subserosa, or muscles) and thus prevented migration of the worms to the heart.

This report describes the results of a test conducted to determine if diethylcarbamazine would prevent the development of experimental infection of *D. immitis* in dogs. It also describes the results of a smaller test conducted in a similar manner with the antimonial compound, sodium antimony III-biscatechol disulfonate,** and the arsenical compound, dichlorophenarsine hydrochloride (Filarsen, research).

Materials and Methods

Pups whelped during the winter were sent, before the advent of the mosquito season, to a nonenzootic area in the mountains approximately 1,200 meters above sea level and about 100 miles from Tokyo. This is the same area where test dogs were kept for previous work with antimony.[3] Approximately 70 houses are well isolated from other villages. Prior to taking the pups to the selected area, all dogs in the village were examined for microfilaria and none was found to be infected.

Pupae of *Aedes togoi*, a suitable intermediate host for *D. immitis*, were collected, raised in the laboratory, and the emerging adult mosquitoes allowed to feed on a known infected dog. The mosquitoes were then transported to the experimental area where the infective larvae were dissected from the mosquitoes and injected subcutaneously into the test dogs.

Since the worms first appear in the heart about 85 days postinfection and the migration appears to be completed within 120 days postinfection,[4] therapy (Table 1) was initiated before 60 days postinfection and the dogs were necropsied not less than 120 days postinfection.

The total dosage (500 mg./lb. of body weight) of diethylcarbamazine in group D was 10 times that of group B and C (50 mg./lb. of body weight) and, even though effective, was admittedly unsuitable for practical use. The objective with group D was to determine whether further studies would be needed in the event of failure with groups B and C. Group A dogs served as controls.

Dichlorophenarsine hydrochloride, given daily as an injection of 1.0 mg. of As per kilogram of body

Received for publication Dec. 8, 1960.

From the Department of Internal Medicine and Parasitology, School of Veterinary Medicine, Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan, where Dr. Kume is professor and head, and Drs. Ohishi and Kobayashi are instructors.

This work was supported in part by a grant from the American Cyanamid Company.

The authors are indebted to Dr. Robert L. Burkhart, manager, Animal Health Development, Agricultural Division, American Cyanamid Company, for interest in materializing this project.

* Caricide, produced by American Cyanamid Co., New York, N.Y.

** Stibophen, produced by Winthrop-Stearns, Inc., New York, N.Y.

TABLE 1—Mode of Treatment for the Control of Dirofilaria immitis in Dogs

Group	No. of dogs	Treatment
A	4	Control
B-1	4	One month after experimental infection, treated with 25 mg. or 1/16 tablet of diethylcarbamazine per pound of body weight, as a single oral dose for two consecutive days.
B-2	2	One month after experimental infection, treated with diethylcarbamazine, 25 mg./lb., as a single injection of 0.125 cc./lb. of the 20% solution for two consecutive days.
C-1	4	Two months after experimental infection, treated with 25 mg. or 1/16 tablet of diethylcarbamazine per pound of body weight as a single oral dose for two consecutive days.
C-2	3	Two months after experimental infection, treated with diethylcarbamazine, 25 mg./lb., as a single injection of 0.125 cc./lb. of the 20% solution for two consecutive days.
D-1	2	Dog 18 treated one day and dog 19 treated three days after experimental infection. Given diethylcarbamazine, 100 mg./lb., as an injection of 0.5 cc./lb. of the 20% solution, divided into three doses daily for five consecutive days.
D-2	2	One month after the experimental infection, treated with diethylcarbamazine, 100 mg./lb., as an injection of 0.5 cc./lb. of the 20% solution divided into three doses daily for five consecutive days.
D-3	2	Two months after the experimental infection, treated with diethylcarbamazine, 100 mg./lb., as an injection of 0.5 cc./lb. of the 20% solution divided into three doses daily for five consecutive days.
E	2	Two months after the experimental infection, treated with dichlorophenarsine hydrochloride, 1 mg. As per kilogram of body weight or 2.2 mg. As per pound of body weight, as a single injection for five consecutive days.
F	2	Two months after the experimental infection, treated with sodium antimony-III-biscatechol disulfonate, 1.5 mg. Sb per kilogram of body weight or 3.3 mg. Sb per pound of body weight, as a single injection for five consecutive days.

TABLE 2—The Results of Prophylactic Treatment of Experimental Infection of Dirofilaria immitis in Dogs

				Worms found in the heart and pulmonary artery						
					Male worms		Female worms			
Group	Dog No.	Infective larvae inoculated	Days from infection to necropsy	Total	Total	Length (cm.) Min.–Max. (Av.)	Total	Length (cm.) Min.–Max. (Av.)	Percentage infection	
A	1	162	129	8	2	9.8–11.9 (10.9)	6	8.3–12.1 (10.6)	4.9	
A	2	82	152	18	8	11.9–14.5 (13.0)	10	14.7–20.2 (17.2)	21.9	20.0
A	3	80	152	41	19	11.4–17.3 (13.4)	22	9.1–17.8 (15.3)	51.3	
A	4	51	152	1	1	14.8	....	.....	2.0	
B-1	5	95	122	0	..	.....	....	.....	0.0	
B-1	6	65	135	3	2	12.9–13.6 (13.3)	1	12.2	4.6	24.1
B-1	7	68	153	18	6	11.2–13.7 (13.0)	12	15.5–19.5 (17.7)	26.5	
B-1	8	81	153	53	31	10.0–16.0 (13.7)	22	9.3–19.5 (16.4)	65.4	18.9
B-2	9	61	123	8	4	10.2–12.2 (10.8)	4	9.8–11.3 (10.4)	13.1	8.4
B-2	10	56	153	2	..	.....	2	16.8–17.4 (17.1)	3.6	
C-1	11	61	130	12	6	7.5–12.0 (10.2)	6	7.2–12.7 (11.5)	19.7	
C-1	12	109	134	10	4	9.2–10.4 (9.9)	6	8.5–12.6 (10.8)	9.2	17.8
C-1	13	60	160	20	12	12.8–16.2 (14.9)	8	17.7–21.6 (19.4)	33.3	
C-1	14	79	162	7	3	9.5–13.5 (11.7)	4	9.3–17.3 (13.3)	8.9	26.4
C-2	15	78	162	33	15	10.7–15.3 (13.7)	18	13.7–21.5 (19.0)	42.3	
C-2	16	111	162	31	16	10.8–15.8 (13.5)	15	12.7–18.7 (16.0)	27.9	37.7
C-2	17	79	165	34	17	11.9–16.0 (13.9)	17	15.2–20.1 (17.6)	43.0	
D-1	18	72	135	7	2	9.1–11.4 (10.3)	5	13.8–15.8 (14.9)	9.7	7.5
D-1	19	58	153	3	2	10.3–10.8 (10.5)	1	15.2	5.2	
D-2	20	61	135	2	1	9.8	1	11.7	3.3	1.7
D-2	21	78	135	0	..	.....	..	.....	0.0	
D-3	22	108	134	4	2	11.1	..	.....	3.7	2.7
D-3	23	107	134	1	..	.....	1	10.7	0.9	
E	24	47	135	1	..	.....	1	10.1	2.1	1.1
E	25	80	135	0	..	.....	..	.....	0.0	
F	26	66	135	0	..	.....	..	.....	0.0	0.7
F	27	77	135	1	..	.....	1	11.9	1.3	

weight for five consecutive days to dogs in group E, had been shown previously to destroy all worms in the heart and pulmonary artery.[5]

Sodium antimony was given to dogs in group F. As reported previously,[3] the microfilaricidal dose of 1.5 mg. of Sb per kilogram of body weight was injected daily for five consecutive days. Since previous work proved that approximately twice the microfilaricidal dose of antimony had successfully prevented the development of worms,[3] a reduced microfilaricidal dose of antimony was tested in the present experiment.

At necropsy, the heart and pulmonary artery were carefully examined for worms to determine if the development of the artificially introduced worms and their resultant migration to the heart were successfully prevented in any of the test groups.

Results and Discussion

Although *Dipetalonema reconditum* and *Dirofilaria repens* are not found in Japan, the microfilariae from the dog used as the source of infection were carefully examined and identified as those of *Dirofilaria immitis*; 22 adult worms of *D. immitis* were found in the heart of the dog at necropsy.

Infection was not completely prevented in dogs of any group (Table 2). All worms found were alive, and there was no significant reduction in size as a result of controlling the infection period. Subcutaneous injections of a solution of diethylcarbamazine caused considerable pain in dogs of groups B-2, C-2, D-1, D-2, and D-3. Nausea and vomiting frequently occurred during the first 30 minutes after the injection of this solution in dogs of groups D-1, D-2, and D-3, but was not observed in dogs of groups B-2 and C-2. No other significant signs of toxicosis were observed.

In our preceding work,[3] twice the microfilaricidal dose of sodium antimonyl pyrogallol sulfonate used here destroyed young worms before they reached the heart. However, the microfilaricidal dose of the compound in this experiment did not destroy all of the young worms before they reached the heart. Neither diethylcarbamazine nor the arsenical completely prevented the young worms from migrating to the heart. Apparently, there was no correlation between the number of infective larvae inoculated and the number of worms found in the heart. No comparisons of effectiveness between groups could be made, since the number of test dogs was inadequate for this purpose. Therefore, the only conclusion at which we could safely arrive was that the therapeutic agents, diethylcarbamazine, sodium antimony-III-biscatechol disulfonate, and dichlorophenarsine hydrochloride are less effective for young worms in the intermediate developmental location than diethylcarbamazine and antimonials are for microfilariae in the blood or arsenicals for worms in the heart and pulmonary artery. Under the conditions of the described method, no completely satisfactory and practical drug dosage destroyed young worms. Further investigations of chemotherapeutic agents are desirable.

References

[1] Garnier, W.: A Progress Report on the Use of Caricide as a Preventive of Canine Filariasis. Lederle Vet. Bull., 13, (1954): 13.

[2] Kume, S.: Chemotherapy of Canine Filariasis. Am. J. Vet. Res., 18, (Oct., 1957): 912–923.

[3] Kume, S.: Prophylactic Therapy Against the Developing Stages of *Dirofilaria immitis* Before Reaching the Canine Heart. Am. J. Vet. Res., 19, (July, 1958): 675–676.

[4] Kume, S., and Itagaki, S.: On the Life-Cycle of *Dirofilaria immitis* in the Dog as the Final Host. Brit. Vet. J., 111, (1955): 16–24.

[5] Kume, S., and Ohishi, I.: Observations on the Chemotherapy of Canine Heartworm Infection with Arsenicals. J.A.V.M.A., 131, (Nov. 15, 1957): 476–480.

[6] Otto, G. F., and Maren, T. H.: Studies on the Chemotherapy of Filariasis. VII. Comparative Review of the Possible Therapeutic Agents Available for Canine and Human Filariasis. Am. J. Hyg., 51, (1950): 385–393.

SUMMARIO IN INTERLINGUA

Studios Additional Super un Therapia Prophylactic Contra le Stadios Evolutive de *Dirofilaria immitis* Ante le Arrivata in le Corde Canin

Tres agentes—diethylcarbamazina, le antimonial disulfonato antimonio-III-biscatecholic a natrium, e le arsenical hydrochloruro de dichlorophenarsina—esseva testate pro lor capacitate de destruer vermes de *Dirofilaria immitis* in lor location de disveloppamento intermediari ante lor arrivata in le corde. Como prophylacticos nulle del compositos esseva completemente satisfactori o practic in le dosages testate.

Use of Cell Culture-Adapted Canine Distemper Virus in the Complement-Fixation Test

David T. Karzon, M.D.; James H. Gillespie, V.M.D.; Robert H. Bussell, Ph.D.

SUMMARY

A complement-fixing (CF) antigen was readily prepared from the Onderstepoort strain of canine distemper virus (CDV) which had been adapted to chicken embryo fibroblast cell culture. The CF system presented no difficulty with anticomplementary effect or nonspecific fixation. The antigen did not cross-react with rabies, *Leptospira canicola*, or infectious canine hepatitis-immune serums. After CDV immunization or infection, CF antibody rose regularly within two to four weeks, paralleling the rise in neutralizing antibody. There was a dichotomy in the persistence of the two antibodies. While neutralizing antibody titers remained elevated or fell slightly over a 6- to 12-month period, the CF antibody dropped to undetectable levels within two to four months after primary infection.

INTRODUCTION

The adaptation of canine distemper virus to whole chicken embryo fibroblast cell culture, with the production of specific cytopathic effects,[5] has provided a source of virus which is high in titer and relatively free of host tissue. Complement-fixing (CF) antigen is readily produced in this system, and this furnished the basis for the development of a test for CF antibody described in the present report. A study of CF antibody response in dogs following vaccination and experimental infection will also be presented.

Received for publication Dec. 2, 1960.

From the Department of Pediatrics and Department of Bacteriology and Immunology, University of Buffalo School of Medicine, Buffalo, and Veterinary Virus Research Institute, Cornell University, Ithaca, N.Y. Dr. Karzon is a Markle Scholar in Medical Science.

This work was supported in part by a grant from the National Foundation.

The authors acknowledge the technical assistance of Frances T. Hall.

Materials and Methods

Virus.—The Onderstepoort strain of canine distemper virus (CDV) which had been adapted to chicken embryo fibroblast cell culture was used for preparation of the CF antigen.[5] The virus had been through 208 passages on the chorioallantoic membrane (CAM) of the chicken embryo and 61 to 62 passages in chicken embryo fibroblasts. The following three strains of CDV were used for vaccination: (1) live virus canine distemper vaccine strain,* (2) live virulent Snyder Hill strain in the form of 10% infected dog spleen, and (3) formalin-inactivated virus** prepared from dog spleen.

Neutralization Tests.—Serum neutralization tests[7] were performed using the inhibition of specific lesions on the CAM inoculated by the method of Gorham.[3] Neutralization end points are expressed as $\log_{10}$ of the reciprocal of the 50% serum dilution.

Cell Culture.—Chicken embryo fibroblast cell cultures were prepared as a trypsinized cell suspension, grown in a medium of 0.25% lactalbumin hydrolyzate and 4% calf serum in Hanks' balanced salt solution. Cells were maintained in 50% bovine amniotic fluid, 2% calf serum in Earle's saline solution. The details have been described in prior publications.[1, 5]

Preparation of antigen.—Chicken embryo fibroblast monolayers, prepared in 4-oz. prescription bottles, were inoculated with 0.5 ml. of undiluted CDV stock. Cytopathic effects were visible by the second day, and cell destruction was far advanced on the third day. The cultures were then placed in a mechanical freezer at −70 C., thawed, and the cells then scraped from the glass. Adequate CF antigen titers were obtained when cultures were harvested between the third and sixth days, and were maximal when almost the entire cell population was involved. The crude whole suspension thus obtained was used for CF antigen. Similarly treated chicken embryo cell culture preparations which had not been inoculated, or had been inoculated with normal chicken embryo tissue, served as control antigen for the CF tests.

Complement-Fixation (CF) Test.—The technique used for the CF test was a modification of that described for influenza virus.[4] The modification consisted of changes in temperature of heat inactivation of serum and volume of reacting mixture, as noted later. All serums were heated at 60 C. for

* Lederle Laboratories, Pearl River, N.Y.

** Virogen, produced by Pitman-Moore Co., Indianapolis, Ind.

TABLE 1—Canine Distemper Virus Complement-Fixation, Box Titration

		Dog serum dilutions*								Controls				
		CDV immune							Normal		Complement (units)			
Antigens	Dilutions*	4	8	16	32	64	128	256	4	Antigen	2.0	1.5	1.0	0.5
Canine distemper virus	2	4**	4	4	4	4	3	1	0	0	0	0	3	4
	3	4	4	4	4	3	3	0	..	..	0	0	1	4
	4	4	4	4	4	2	1	0	..	..	0	0	1	4
	6	4	4	4	4	±	0	0	..	..	0	0	0	4
	8	4	4	4	2	0	0	0	..	..	0	0	0	3
	10	4	4	3	0	0	0	0	..	..	0	0	0	3
Tissue culture control	2	0	0	0	0	0	0	0	0	0	0	0	0	3
Serum control	..	0	..	..	..	..	..	..	0	..	..	..	..	..

* Reciprocal of serum or antigen dilutions. ** 4 = complete fixation.

30 minutes. A preliminary titration of commercial guinea pig complement* was performed in the presence of antigen, and two units of complement were employed. The diluent was 0.15 M NaCl solution, containing 0.0002 M Ca^{++} and 0.0004 M Mg^{++}. One-tenth milliliter of serum, 0.1 ml. of antigen (2 units), and 0.1 ml. of complement were incubated together at 4 C. for 18 hours, followed by ten minutes at 37 C., prior to addition of 0.2 ml. of 1% sensitized sheep cells. Following this, the tubes were maintained at 37 C. and the readings were made at 30 minutes. The final volume was 0.5 ml. The sheep cells were sensitized by treatment for ten minutes at room temperature with an equal volume of rabbit hemolysin containing two hemolytic units.

Antibody titers are expressed throughout the paper as the reciprocal of the serum dilution. Serum titers of less than 4 or, in some instances, less than 2, which were the lowest dilutions tested, were considered to represent nondetectable levels in the present study.

Results

Properties of CF Antigen.—The crude whole suspension of chicken embryo fibroblast cell culture inoculated with CDV, as described, had an infectivity titer of approximately 7×10^5 plaque-forming units per milliliter and a CF antigen titer of 4 to 8. No difficulty was encountered with anticomplementary activity or nonspecific fixation using viral or control antigens. A typical box titration, including specific CDV and control tissue culture antigens, is shown (Table 1). The CF antigen was stored at −70 C., without significant loss of titer, for at least 4½ months. The antigen was relatively heat labile, being inactivated completely at 56 C. for 30 minutes and losing approximately half its titer when held at 37 C. for six hours.

Complement-Fixing Antibody Response in CDV-Infected and Immunized Dogs.—Dogs approximately 12 to 16 weeks of age and free of CDV neutralizing antibody were used. Three groups of dogs, representing three types of CDV immunization or infection, were studied: In group 1, 5 dogs were given a single dose of the chicken-embryo strain, 1.0 ml. subcutaneously. All dogs remained well. In group 2, 2 dogs were

* Carworth Farms, New City, Rockland County, N.Y.

TABLE 2—Immunization with Chicken Embryo Adapted Canine Distemper Virus*—Group 1

	Complement-fixation **					Neutralization †				
Days	Dogs: 680	689	690	691	682	Dogs: 680	689	690	691	682
0	<4	<4	....	....	<4	<0.5	<0.5	....	0.7	....
15	....	....	<4	....	....	....	....	0.7	....	....
23	16	....	....	....	8	3.3	....	....	....	3.3
29	....	4	8	4	....	....	3.7	3.6	3.6	....
34	4	....	....	....	16	3.4	....	....	....	3.7
57–61	4	4	8	16	8	3.3	3.6	3.4	3.4	3.3
85–89	4	4	8	8	8	....	3.3	3.3	3.3	3.3
127–145	<4	....	<4	<4	4	3.3	....	3.5	3.3	3.4
183–338	<4	<4	<4	<4	<4	2.8	2.9	3.1	2.9	3.3
420–711	<4	<4	<4	<4	<4	2.5	2.9	2.9	2.4	2.6

* Lederle Laboratories, Pearl River, N.Y. ** Expressed as reciprocal of serum dilution. All leaders (....) in the table represent intervals in which no samples were tested. † Expressed as $\log_{10}$ of reciprocal of 50% serum dilution.

TABLE 3—Experimental Canine Distemper Virus Infection (Snyder Hill Strain)—Group 2

Days	Complement-fixation **				Neutralization †			
	Dogs: 342*	343*	345‡	346‡	Dogs: 342*	343*	345‡	346‡
0	<4	<4	<2	<2	<0.5	<0.5	<0.5	<0.5
13	<4	<4	....	....	0.7	<0.5	<0.5	<0.5
17	....	<4	<4	<4	....	1.6	<0.5	<0.5
25	16	<4	4	<4	3.0	2.2	1.7	1.1
59	8	<4	16	8	3.0	2.7	2.9	3.4
88	16	4	32	32	2.9	2.7	2.9	2.9
116	4	....	4	8	2.7	2.9	2.9	3.0
145	<4	....	<4	<4	2.4	2.9	2.9	2.9
172	<4	<4	8	16	2.4	2.9	2.8	2.9
254	....	....	32	8	....	....	....	....
310	16	<4	8	....	3.4	3.4	3.4	3.0
429	....	....	16	<4	2.7	3.7	3.3	2.9
551	8	<4	8	....	2.7	2.6	3.4	3.0
775	4	<4	<4	<4	2.8	2.4	2.7	2.7

* Infected with canine distemper virus. ** Expressed as reciprocal of serum dilution. All leaders (....) in the table represent intervals in which no samples were tested. † Expressed as $\log_{10}$ of reciprocal of 50% serum dilution. ‡ Exposed to infected dogs.

given the Snyder Hill strain, 1.0 ml. intravenously. Two dogs were housed with them to serve as contact controls. All 4 dogs became clinically ill. In group 3, 4 dogs were given three injections of formalin-inactivated dog spleen virus at biweekly intervals, and their immunity was challenged 3½ months later with Snyder Hill virus. Groups 1 and 3 were maintained in isolation units throughout the course of the experiment. The dogs in group 2 were maintained in isolation for six months and thereafter kept in a barn where complete isolation technique was impractical.

The 5 dogs in group 1, given the live virus vaccine, developed CF antibody by the 23rd to the 29th day (Table 2). The CF antibody decreased to nondetectable levels within three months and remained at this level during the two-year duration of the experiment. The neutralizing antibody response reached peak titers within the first month and decreased somewhat over the ensuing two years.

The antibody response of the 2 dogs in group 2 infected with virulent Snyder Hill virus, as well as 2 exposed littermates, is shown (Table 3). All 4 dogs developed a CF antibody titer which subsequently decreased to less than 4 by five months. Three dogs had a second rise in CF antibody toward the end of the first year, at a time when they were housed outside the isolation area. The latter CF responses were not accompanied by known illness, but there was a slight rise of neutralizing antibody at this time as well.

The CF antibody response (group 3) fol-

TABLE 4—Immunization with Formalin-Inactivated Infected Dog Spleen* and Challenge with Canine Distemper Virus (Snyder Hill Strain)—Group 3

Days	Complement-fixation **				Neutralization †			
	Dogs: 738	739	740	742	Dogs: 738	739	740	742
0	<4	<4	<4	<4	<0.5	<0.5	<0.5	<0.5
14	<4	32	<8	4	1.2	0.9	1.4	0.6
29	16	16	16	16	1.8	2.5	2.2	1.4
49	8	8	<4	8	1.0	2.4	1.5	1.5
61	....	<4	....	4	....	....	....	1.6
75	<4	<4	<4	<2	....	1.1	....	0.9
104‡	<4	<2	<4	<4	<0.5	<0.5	<0.5	<0.5
106	<4	<4	....	<4	<0.5	<0.5	<0.5	<0.5
108	....	....	<4	16	2.4	1.8	2.4	2.4
110	32	....	....	....	4.4	3.0	3.8	4.6
112	....	64	128	128	5.6	4.6	5.2	5.3
115	....	64	.....	128	5.0	6.0	6.0	5.0
118	64	64	128	.....	6.8	5.0	5.4	5.4
132	32	16	64	64	6.4	5.5	5.2	6.5
193	32	16	32	64	6.0	5.5	6.4	6.4
285	16	16	16	32	5.0	6.7	6.5	5.2
307	....	16	....	....	....	6.7	....	....

* Virogen, Pitman-Moore Co., Indianapolis, Ind. ** Expressed as reciprocal of serum dilution. All leaders (....) in the table represent intervals in which no samples were tested. † Expressed as $\log_{10}$ of reciprocal of 50% serum dilution. ‡ Immunity of dogs challenged with virulent CDV (strain Snyder Hill) on day 104.

lowing inoculation with inactivated CDV of spleen origin was prompt, being detectable in 2 dogs by the 14th day (Table 4). However, the CF titers decreased rapidly and were not detectable at 2½ months. At 3½ months, the immunity of these dogs was challenged with Snyder Hill virus. They had a febrile response only, and did not develop the classical clinical signs of distemper. Within four to eight days, all animals developed unusually high titers (64 to 128) of CF antibody. The titers decreased, but remained at detectable levels for the ensuing seven months of observation. In this group of dogs, there was only a modest neutralizing antibody response (1.6 to 2.5 log) to the initial immunization, but a rapid and exaggerated response (6.5 to 6.8 log) to Snyder Hill challenge.

Complement Fixation Antibody in Random Street Dogs.—Twenty-nine dogs of various ages and backgrounds were studied for neutralizing and CF antibody. Of 23 dogs with neutralizing antibody, 15 had CF antibody in titers of 4 to 32, while 8 did not have CF antibody at a dilution of 4. The 6 dogs without neutralizing antibody also had no CF antibody.

Specificity of CDV CF Test.—Dogs immunized against three unrelated infectious agents were tested for the development of CDV CF antibody. Dogs immunized against rabies (chicken embryo adapted Flury strain), *Leptospira canicola* (maintained in hamsters), and infectious canine hepatitis (Cornell strain 1) did not acquire CDV CF antibody.

Discussion

In the past, CDV CF antigens have been prepared using aqueous [6] or benzene [8] extracts from infected ferret or dog tissues. The difficulties of this system, studied in detail by Mansi,[6] included nonspecific fixation by normal tissue and a variable procomplementary effect of distemper and control antigens. Distemper virus strains adapted to the chicken embryo failed to yield CF antigen in extracts of CAM, yolk sac, or embryo.[6] However, a suitable antigen could be produced from infected CAM by sonic disruption.[7] In contrast, the present chicken embryo fibroblast cell culture antigen was readily prepared without special techniques. It was used in a dilution of 1:2 or 1:3 of crude tissue culture fluid, and anticomplementary activity was not a problem. Normal dog serums in the presence of CDV antigen or tissue culture control antigen did not fix complement nonspecifically. Canine antiserums prepared against *L. canicola*, rabies, and infectious canine hepatitis did not react with CDV CF antigen.

Complement-fixation antibody developed regularly two to four weeks following immunization or experimental infection, paralleling the rise in neutralizing antibody. However, the CF antibody persisted for only two to four months in all groups. This affirms the data obtained with other distemper CF systems.[6] Reinoculation of dogs originally given inactivated virus and subsequently having lost all measurable amounts of CF or neutralizing antibody (group 3) produced a typical secondary or anamnestic response. Within a brief period of four days an unusually high CF and neutralizing antibody response occurred, and the CF titers remained elevated for the ensuing seven months. Three dogs with experimental Snyder Hill virus infection (group 2) had a secondary rise in CF antibody long after the primary infection. There was also a slight elevation of neutralizing antibody titer in some of the dogs at this time. This raises the question of a possible unrecognized reinfection with CDV, but no information is available on this point from these experiments. Simultaneous study of the CF and neutralizing antibodies may contribute to knowledge of the role of repeated exposure to infection in the maintenance of CDV neutralizing antibody and resistance to the naturally occurring clinical disease. The 29 street dogs selected at random represented three possible combinations of the two antibodies. Fifteen dogs had neutralizing and CF antibody, which may be interpreted as indicating recent infection or immunization. Eight dogs with neutralizing but no CF antibody may represent remote infection. In 6 dogs in which both antibodies were absent, there was no evidence of previous CDV infection.

References

[1] Dulbecco, R.: Production of Plaques in Monolayer Tissue Cultures by Single Particles of an Animal Virus. Proc. Nat. Acad. Sci. (U.S.A.), 38, (1952): 747–752.

[2] Gillespie, J. H., Baker, J. A., Burgher, J., Robson, D., and Gilman, B.: The Immune Response of Dogs to Distemper Virus. Cornell Vet., 48, (1958): 103–126.

[5] Gorham, J. R.: A Simple Technique for the Inoculation of the Chorioallantoic Membrane of Chicken Embryos. Am. J. Vet. Res., 18, (July, 1957): 691–692.

[6] Jensen, K. E.: Influenza. In *Diagnostic Procedures for Virus and Rickettsial Diseases*. Am. Public Health Assoc., New York, N.Y., (1956): 241–262.

[7] Karzon, D. T., and Bussell, R. H.: Cytopathic Effect of Canine Distemper Virus in Tissue Culture. Science, 130, (1959): 1708–1709.

[8] Mansi, W.: The Value of the Complement-Fixation Test in the Study of Canine Distemper Complex and Rubarth's Disease. J. Comp. Path. & Therap., 65, (1955): 291–308.

[9] Morris, J. A., Aulisio, C. G., and McCown, J. M.: A Complement-Fixation Test for Canine Distemper. Cornell Vet., 45, (1955): 182–189.

[10] Savan, M., and Brandly, C. A.: Complement-Fixation Test for Fox Distemper. Proc. Book, AVMA (1950): 211–216.

SUMMARIO IN INTERLINGUA

Le Uso de Cytoculturalmente Adaptate Virus de Maladia de Can in le Test a Fixation de Complemento

Un antigeno complemento-fixante (CF) esseva prestemente preparate ab le racia Onderstepoort del virus de maladia de can (VMC) que habeva essite adaptate a un cytocultura de fibroblastos de embryon de gallina. Le systema CF presentava nulle difficultate per effectos anti-complementari o per fixation non-specific. Le antigeno non entrava in reaction cruciate con ravie, *Leptospira canicola*, o seros immun pro infectiose hepatitis canin. Post immunisation o infection a VMC, le anticorpore CF montava regularmente intra duo a quatro septimanas, in parallela al augmento del neutralisante anticorpore. Esseva observate un dichotomia in le persistentia del duo anticorpores. Durante que le titros de neutralisante anticorpore remaneva elevate o declinava levemente in le curso de un periodo de 6 a 12 menses, le anticorpore CF declinava usque a non-detegibile nivellos intra duo a quatro menses post le infection primari.

A Technique for Concentrating *Mycobacterium paratuberculosis* Present in Intestinal Mucosa

Aubrey B. Larsen, D.V.M., M.S., and Richard S. Merkal, B.S.

SUMMARY AND CONCLUSIONS

The efficacy of sodium hydroxide, papain, trypsin, and pepsin for separating *Mycobacterium paratuberculosis*, the causative agent of Johne's disease, from intestinal tissue was studied.

The best results were obtained with trypsin digestion, which provided a rapid practical method of decreasing the volume of tissue containing small numbers of bacilli. Obviously, such a concentration of bacilli facilitated the demonstration of *Myco. paratuberculosis* in specimens from suspect animals.

INTRODUCTION

The causative agent of Johne's disease, *Mycobacterium paratuberculosis*, is often difficult to identify in stained smears prepared from intestinal mucosae of suspect animals. Laboratory diagnostic methods are needed that will increase the chances of finding the bacillus when it occurs in small numbers in specimens.

Methods have been described for concentrating *Mycobacterium tuberculosis* when it is present in sputum, aspirated gastric contents, and urine. Generally, the methods use enzymes [2,8,10] or alkalies [4,7,9] for digesting the mucus and tissue. The bacilli are then concentrated by centrifugation.

In preparing lepromin, Lew *et al.* [5,6] used 0.5% trypsin solution for digesting tissue containing *Mycobacterium leprae*. This method facilitated the detection of bacilli when the diagnosis of leprosy was doubtful. Khanolkar and Rajalakshmi [3] used a petroleum ether flotation procedure for concentrating *Myco. leprae*. Dharmendra and Mukherjee [1] extracted *Myco. leprae* from minced tissue by using chloroform.

Our laboratory studies showed that digesting intestinal mucosa with 0.5% trypsin solution for one hour at 22 C. facilitated the concentration of *Myco. paratuberculosis* for use in cultural techniques. Because of these results and the proved value of digestion methods in diagnosing tuberculosis and leprosy, various digestion techniques were studied. The best method was selected. Comparative studies were made on microscopic detection of *Myco. paratuberculosis* in undigested and digested intestinal mucosa. These studies were accomplished by using normal intestinal mucosa with added bacilli, intestinal mucosa from an ox infected with Johne's disease, and intestinal specimens from suspect animals.

Materials and Methods

The Ziehl-Neelsen staining procedure, described by Kolmer *et al.*,[4] was used throughout the experiments. One hundred fields on each preparation were examined under oil immersion.

Intestinal mucosa was scraped from the cecum of a healthy bovine animal, thoroughly mixed,* and examined for acid-fast bacilli. Since none were found, the material was used as a diluent in experiments 2 and 3.

Intestinal mucosa from an ox infected with *Myco. paratuberculosis* was similarly treated and examined. A few typical acid-fast bacilli were found in most fields, and this tissue was used in experiments 1 and 3.

A stock culture of *Myco. paratuberculosis* strain 18 was used in experiment 2.

The following solutions were prepared, using distilled water: (1) a 0.5% solution of trypsin (1–110 Pfanstiehl); (2) a pepsin solution consisting of 1 Gm. of pepsin powder N.F. (Fisher) and 0.15 ml. concentrated HCl in 100 ml. of water; (3) a 1.0% solution of papain (Difco); and (4) a 4.0% solution of NaOH.

Experiment 1.—In a preliminary trial, infected intestinal mucosa diluted 1:125 with normal mucosa was tested in 2-ml. portions as follows: Portion 1 was mixed with 100 ml. of trypsin solution, adjusted to pH 9 with NaOH solution, and stirred for one hour at 22 C. Portion 2 was mixed with 100 ml. of pepsin solution and stirred for four hours at 38 C.[2] Portion 3 was placed in 50 ml. of

Received for publication Jan. 1, 1961.

From the Regional Animal Disease Research Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, USDA, Auburn, Ala.

* In a Mickle machine, manufactured by H. Mickle, Gomshall, Surrey, England, and sold in the United States by Brinkman, Great Neck, New York. This machine mixes by shaking the materials 60 times per second in a container with glass beads.

papain solution and stirred for 15 minutes at 38 C.[3] Portion 4 was mixed with 100 ml. of 4% NaOH solution,[4] stirred for one hour at 22 C., and then neutralized with HCl.

All four specimens were centrifuged for one hour at 2,000 r.p.m. (1,300g). The supernatant fluid was discarded, and two smears prepared from each sediment were stained and examined.

Experiment 2.—Eighty milligrams of moist *Myco. paratuberculosis* was added to 4 Gm. of normal intestinal mucosa to make a concentration of bacilli of 1:50. The mixing machine dispersed the bacilli throughout the mucosa. One gram of the mixed material was added to 3 Gm. of normal mucosal scrapings and mixed in the machine. Serial dilutions were made in this fashion until the following concentrations or proportions of *Myco. paratuberculosis* to diluent were obtained: 1:200, 1:800, 1:3,200, 1:12,800, 1:51,200, and 1:204,800. Two smears were prepared from each dilution for controls. The remaining 3 Gm. of each dilution of bacilli with mucosa was mixed with 150 ml. of the trypsin solution, adjusted to pH 9, and stirred on a magnetic stirrer for one hour. The mixture was then centrifuged for one hour at 2,000 r.p.m. (1,300g.), the supernatant fluid was discarded, and two smears were prepared and examined from each sediment.

Experiment 3.—One Gm. of infected intestinal scrapings was added to 1 Gm. of normal mucosa and machine-mixed. Serial dilutions were made from this material until the following concentrations were obtained: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512. Two smears were prepared from each dilution for controls. The remainder of each concentration was mixed with trypsin solution, digested, and centrifuged as previously described. Two smears were prepared and examined from each sediment.

Experiment 4.—Forty intestinal specimens from animals in infected herds were first examined by the following routine diagnostic method: Two smears were prepared from the ileocecal valve, two from the cecum (each about 6 inches on either side of the valve), and one from a lymph gland adjacent to the valve. These smears were stained, and 60 fields were examined on each slide.

For the digestion procedure, 1 Gm. of mucosa was removed from the ileocecal valve and digested in 50 ml. of trypsin solution. The remaining procedure was identical to that of the previous experiments. Two smears were prepared and examined from each sediment.

Results

Experiment 1.—Several methods of digesting intestinal mucosa were compared to determine the best method of demonstrating *Myco. paratuberculosis* (Table 1). Trypsin and sodium hydroxide were superior to pepsin and papain as digestion agents. The sediment from trypsin digestion was creamier than that from sodium hydroxide, and more uniform smears were obtained. This uniformity could account for trypsin producing slightly higher numbers of positive fields than sodium hydroxide. Also, since trypsin digestion did not require washing or neutralization, it was studied further in experiments 2, 3, and 4.

TABLE 1—A Comparison of Four Agents Used to Digest Intestinal Scrapings Containing Mycobacterium paratuberculosis

Agent used	No. of slides	No. of fields containing acid-fast bacilli*
Trypsin	2	30
Sodium hydroxide	2	25
Pepsin	2	4
Papain	2	0
Undigested mucosa	2	4

* 100 fields were examined on each slide.

Experiment 2.—*Mycobacterium paratuberculosis* bacilli had been added to normal intestinal mucosa, and comparisons were made of the concentrations (Table 2). When the concentration of bacilli was 1:3,200 or less, they could be demonstrated more readily in smears prepared from the trypsin-digested material than in those from the undigested mucosa.

TABLE 2—The Effect of Trypsin Digestion on the Demonstration of Mycobacterium paratuberculosis in Intestinal Mucosa to Which Bacilli Were Added

Dilution of the bacilli in normal scrapings	No. of positive fields in 200 examined	
	Undigested scrapings	Digested scrapings
1:50	200	Not examined
1:200	95	Not examined
1:800	25	156
1:3,200	6	144
1:12,800	0	25
1:51,200	1	2
1:204,800	0	0

TABLE 3—The Effect of Trypsin Digestion on the Demonstration of Mycobacterium paratuberculosis in Infected Intestinal Mucosa

Dilutions of infected scrapings	No. of positive fields in 200 examined	
	Undigested scrapings	Digested scrapings
1:2	188	Not examined
1:4	59	116
1:8	87	121
1:16	26	156
1:32	13	63
1:64	8	88
1:128	1	57
1:256	1	6
1:512	0	5

TABLE 4—Comparative Diagnosis of Intestinal Specimens from Cattle Suspected of Harboring Mycobacterium paratuberculosis

No. of specimens	Results of routine laboratory examination (No. of positive fields*)	No. of positive fields found in trypsin-digested materials**
25	0	0
12	0	1 to 31
3	1 to 2	4 to 14

* 300 fields examined. ** 200 fields examined.

Experiment 3.—Normal mucosa had been diluted with infected mucosa. When the concentration of infected mucosa was 1:4 or less, the bacilli were found more readily in smears from the digested material than in those from the undigested mucosa (Table 3). When the concentration of infected mucosa was 1:512, the bacilli were found only in the digested material.

Experiment 4.—Two methods of examination were compared on 40 intestinal specimens from animals in infected herds (Table 4). In 25 specimens, no bacilli were found by either method. In 12 specimens, no bacilli were found in the undigested specimens, but they were in smears prepared from the digested material. In three specimens, the bacilli were found by both methods.

References

[1] Dharmendra, and Mukherjee, N. M.: A Simple Method of Concentration of Leprosy Bacilli from "Closed" Cases of Leprosy. Leprosy in India, 24, (1952): 169–177.

[2] Gerundo, M.: Sputum Concentration for the Culture of Tubercle Bacillus. J. Lab. & Clin. Med., 28, (1942): 328.

[3] Khanolkar, V. R., and Rajakakshmi, K.: A Method for Concentration of Acid-Fast Bacilli in Skin Biopsies from Leprosy Patients. Leprosy in India, 24, (1952): 47–61.

[4] Kolmer, J. A., Spaulding, E. H., and Robinson, H. W.: Approved Laboratory Technique. Appleton-Century-Crofts, Inc., New York, 1951.

[5] Lew, J., and Carpenter, C. M.: The Separation of *M. Leprae* from Tissues by Enzyme Digestion. Am. Rev. Tuberc., 74, (1956): 152.

[6] Lew, J., and Chung, M.: The Bacteriological Diagnosis of Early Leprosy by the Trypsin Digestion Method. Internat. J. Leprosy, 27, (1959): 360–365.

[7] Petroff, S. A.: A New and Rapid Method for the Isolation and Cultivation of Tubercle Bacilli Directly from the Sputum and Feces. J. Exptl. Med., 21, (1915): 38–42.

[8] Sullivan, N. P., and Sears, H. J.: A Simple Technique for Concentrating Tubercle Bacilli in Sputum. J. Lab. & Clin. Med., 24, (1939): 1093–1095.

[9] Uhlenhuth, P., and Zylander, A.: Antiformin ein Bacterienan Flosendes Desinfektionsmittel. Berl. Klin. Wchnschr., 45, (1908): 1346.

[10] Vogt, A. B., Zappasodi, P., and Long, E. R.: Bacillary Counts in Sputum. A Quantitative Method for Determining the Number of Tubercle Bacilli in Sputum. Am. Rev. Tuberc., 41, (1940): 481–493.

SUMMARIO IN INTERLINGUA

Un Technica pro Concentrar le *Mycobacterium paratuberculosis* Que Es Presente in Mucosa Intestinal

Esseva studiate le efficacia de hydroxydo de natrium, de papaina, de trypsina, e de pepsina in le separation de *Mycobacterium paratuberculosis* (le agente etiologic de morbo de Johne) ab tissus intestinal. Le melior resultatos esseva obtenite per medio de digestion a trypsina. Iste agente provideva un rapide methodo practic pro reducer le volumine de tissu continente un miere numero de bacillos. Obviemente, un tal concentration facilitava le demonstration de *Myco. paratuberculosis* in specimens ab animales suspecte.

Association Among Members of the Genus *Metastrongylus* Molin, 1861 (Nematoda : Metastrongylidae)

S. A. Ewing, D.V.M., M.S., and A. C. Todd, Ph.D.

SUMMARY AND CONCLUSIONS

Three experiments were conducted to prove an apparent association phenomenon between *Metastrongylus apri* and *Metastrongylus pudendotectus* previously reported by the writers as an observation in natural lungworm populations. Three different sizes (100, 400, and 6,000 larvae) of inoculums were used singly and in combination (i.e., *M. apri* alone, *M. pudendotectus* alone, and a combination of the two). In single species exposures, worms were not recovered unless the number of larvae given exceeded 400, but in dual exposures, as few as 100 larvae were sufficient to produce infections. The percentage of the inoculum recovered as adults in the dual exposures, when 6,000 larvae were given, was overwhelming as compared with the single exposures. Furthermore, maturation of the worms was hastened in the dual exposures. *Metastrongylus pudendotectus* is less able than *M. apri* to successfully parasitize swine when present alone, as indicated by the small percentage of the inoculums recovered and the relatively smaller size of the parasites. No basis for an explanation of the phenomenon was apparent from these experiments. The association apparently is not obligatory but is certainly advantageous to both species.

INTRODUCTION

In a recent survey, the species and sex ratios of metastrongyles in naturally infected swine were determined and an apparent "association" between *M. apri* and *M. pudendotectus* was found. In 76% of animals examined, the two species were associated to the exclusion of the third species, *Metastrongylus salmi*, and in 11% all three species were present. *Metastrongylus apri* and *M. pudendotectus* occurred as single species in only 11% and 1% of the cases, respectively.[1]

A series of experiments was completed to determine whether the association found between *M. apri* and *M. pudendotectus* represented a chance distribution or was characteristic of natural infections.

Materials and Methods

With one exception, swine used in these studies were farrowed in strict isolation by dams free of helminths, were weaned at 8 weeks of age, and were exposed subsequently to infective *Metastrongylus* larvae. In the one case, weanling swine were purchased and maintained under circumstances excluding possible accidental exposure to metastrongyles. Feces from these latter animals were examined daily for approximately one month before the animals were exposed to infective lungworm larvae. As an added assurance that they were lungworm-free, daily fecal examinations were continued until the first metastrongyle eggs were recovered 27 days postexposure, *i.e.*, 54 days after confinement.

Metastrongylus larvae to which swine were exposed were from laboratory-infected earthworms of the genus *Eisenia*, and all larvae used in a specific experiment were approximately the same age. The eggs to which the earthworms were exposed came from lungworms collected from naturally infected swine.

A 5-Gm. sugar flotation technique was utilized in recovery of eggs from the feces of experimental swine. All animals were killed at the end of the respective experiments, and necropsies were made immediately. Magnification was employed when examining the respiratory apparatus for helminths.

Experimental Procedure and Results

Experiment 1.—Pig 1 was given approximately 100 infective *M. apri* larvae; pig 2, an equivalent number of *M. pudendotectus*. Pigs 3, 4, and 5 were each given approximately 50 *M. apri* and 50 *M. pudendotectus*. Thus, each of the 5 swine was exposed to approximately 100 third-stage *Metastrongylus* larvae.

Received for publication Dec. 12, 1960.

From the Department of Veterinary Science, University of Wisconsin, Madison. Published with the approval of the director, Wisconsin Agricultural Experiment Station, as paper No. NS 305.

Supported by a grant from the National Institutes of Health.

Transient coughs of mild intensity developed in all 5 animals within a week following exposure. Daily fecal samples remained negative until 27 days postexposure. Animals 4 and 5 both passed larvated metastrongyle eggs on the same day and erratic numbers of eggs persisted in the feces until slaughter. None of the remaining 3 animals was ever found to be shedding helminth eggs.

Necropsy was made 35 days postexposure. The number and sex distribution of the two helminth species recovered are shown (Table 1). The 3 swine given the dual exposure consisting of *M. apri* and *M. pudendotectus* harbored adult metastrongyles at slaughter, but none was recovered from the other 2 swine.

Experiment 2.—Four swine were each exposed to approximately 400 third-stage *Metastrongylus* larvae. Swine 1 and 2 were each given approximately 200 third-stage larvae of *M. apri* and an equal number of *M. pudendotectus*. Pig 3 was given approximately 400 *M. pudendotectus*; pig 4, an estimated equal dose of *M. apri*.

At 29 days postexposure, swine 1 and 2 began shedding *Metastrongylus* eggs. Samples from these 2 animals remained positive until the time of slaughter. Neither pig 3 nor 4 was found to be passing helminth eggs.

Both swine given the dual exposure harbored adult metastrongyles at slaughter, whereas none was recovered from the other 2 animals which had been exposed to single species. Table 2 relates the magnitude of the infections as shown at necropsy 56 days postexposure. Areas of emphysema were observed in the diaphragmatic lobes of animals 1 and 2; no gross lesions were observed in animals 3 and 4.

Experiment 3.—Each of 6 littermate swine was exposed to approximately 6,000 infective metastrongyle larvae but, by pairs of animals, three qualitatively different exposures were made. One pair (swine 5 and 6) were given *M. apri* only; another pair (swine 3 and 4). *M. pudendotectus* only; and the remaining pair (swine 1 and 2), approximately equal numbers of both species.

All 6 swine developed severe signs of respiratory distress within a week following exposure. Coughing was general in the group, and no particular animal had an exceptional degree of affliction. Coughing was easily elicited in all 6 animals by exercise until the time of slaughter. Terminally, the pair given the dual exposure

TABLE 1—Summary of Helminths Recovered at Necropsy from Swine Experimentally Exposed to Approximately 100 Third-Stage Metastrongyle Larvae

Animal No.	Exposure	*Metastrongylus apri*		*Metastrongylus pudendotectus*		Total
		Male	Female	Male	Female	
1	*M. apri*, only	....	....	....	....	....
2	*M. pudendotectus*, only	....	....	....	....	....
3	*M. apri* and *M. pudendotectus* (equal numbers)	19	8	12	8	47
4	*M. apri* and *M. pudendotectus* (equal numbers)	23	8	11	5	47
5	*M. apri* and *M. pudendotectus* (equal numbers)	14	11	8	5	38

TABLE 2—Summary of Helminths Recovered at Necropsy from Swine Experimentally Exposed to Approximately 400 Third-Stage Metastrongyle Larvae

Animal No.	Exposure	*Metastrongylus apri*		*Metastrongylus pudendotectus*		Total
		Male	Female	Male	Female	
1	*M. apri* and *M. pudendotectus* (equal numbers)	10	19	5	8	42
2	*M. apri* and *M. pudendotectus* (equal numbers)	54	104	10	17	185
3	*M pudendotectus*, only	....	.....	....	....	.....
4	*M. apri*, only	....	.....	....	....	.....

TABLE 3—Summary of Helminths Recovered at Necropsy from Swine Experimentally Exposed to Approximately 6,000 Third-Stage Metastrongyle Larvae

Animal No.	Exposure	*Metastrongylus apri*		*Metastrongylus pudendotectus*		Total
		Male	Female	Male	Female	
1	*M. apri* and *M. pudendotectus* (equal numbers)	686	1,619	181	263	2,749
2	*M. apri* and *M. pudendotectus* (equal numbers)	445	882	58	96	1,481
3	*M. pudendotectus*, only	.....	.....	4	14*	18
4	*M. pudendotectus*, only	.....	.....	8	26*	34
5	*M. apri*, only	29	63**	.....	....	92
6	*M. apri*, only	105	268**	.....	....	373

* All sexually immature. ** Many sexually immature.

(swine 1 and 2) had more protracted seizures than either of the other two pairs.

The first lungworm eggs were recovered 30 days postexposure. Animals 1 and 2, exposed to both species, had positive fecal samples from 30 days postexposure daily to the time of slaughter. Terminally, 1 animal (pig 5) of the pair given only *M. apri* larvae also was shedding small numbers of lungworm eggs. None of the remaining 3 animals ever was found to be shedding helminth eggs.

At necropsy 40 days postexposure, the gross lesions were principally those of chronic pneumonia and emphysema. Although emphysematous lesions were more pronounced in animals 1 and 2, chronic pneumonia appeared equally severe in all members of the group. Emphysema was moderate in 1 animal (pig 6) of the pair given *M. apri*; it was essentially absent from the other animal (pig 5) of the pair as well as from the pair exposed to *M. pudendotectus*.

Table 3 details the helminth recoveries made at necropsy.

Discussion

The natural lungworm population in Wisconsin swine can be described with considerable exactness. Goldsby and Todd [2] reported a 56% prevalence of *Metastrongylus* in market-weight swine examined at random in the years 1955 to 1956. These populations varied between 1 to 404 specimens and averaged 50.1. Ewing and Todd [1] described the natural lungworm population found in 100 sets of swine lungs selected on the basis of presence of the parasites. In the latter study, 54.2 worms comprised the average population in the years 1958 to 1959, a figure remarkably similar to that obtained in 1955 to 1956.

Because the lungworms had not been identified in the earlier survey,[2] the data were collected during the second survey.[1] It was found that the lungworm population in Wisconsin typically was comprised of two species, *M. apri* and *M. pudendotectus*. That is to say, the two species were associated in 76% of the infections; in 11% of the infections, *M. apri* and *M. pudendotectus* were associated with *M. salmi*. On the other hand, populations consisted of a single species only in 13% of the infections. In populations of single species, *M. apri* occurred alone in 11%, *M. pudendotectus* in 1%, and *M. salmi* in 1%.

The success of a parasite perhaps can be measured in several ways. A question concerning association of one species of parasite with others and its success in natural populations has received little previous attention.

At least one survey, of natural helminthic infections in Tennessee chickens, described populations and arrayed the average population according to numbers of species present.[4] The population of *Ascaridia galli*, for example, ranged from 4.6 worms when *A. galli* was the only helminth present, through 4.4, 4.9, 6.9, and 8.4 when one, two, three, or four other species were present. The progression is statistically improbable. On the other hand, since the total helminth population increased numerically and improbably as the number of species comprising the population increased, the above figures may not necessarily reflect association among species; they may reflect sanitary conditions in a given poultry flock.

The three laboratory experiments now reported appear to support an association among lungworm species, specifically *M. apri* and *M. pudendotectus*. In the first trial, adult parasites developed only in the

3 animals exposed to two species. The second trial confirmed the first trial in that adult parasites developed only in the 2 animals exposed to two species. A variant was introduced into the second trial when total numbers of larvae used in exposure were increased from 100 to 400 larvae.

It is considered that the third trial supports the findings in the first two trials. While a single species of lungworm was able to develop in the 4 animals exposed to single species, the success of each species was significantly enhanced when it was associated at exposure with the second species (Table 3). Thirty-five per cent of the larvae developed to maturity in hosts given dual exposures; less than 1% of the larvae developed in pigs exposed to *M. pudendotectus* only, and none reached sexual maturity. Somewhat less than 4% of the larvae were able to develop in pigs exposed to *M. apri* only, and many of these had not achieved sexual maturity prior to necropsy.

Schwartz and Alicata [3] observed a single case in which *Choerostrongylus pudendotectus (M. pudendotectus)* developed in the absence of other species. In another instance, a single worm was recovered five days after exposure to a single species. They found that *Metastrongylus elongatus (M. apri)* developed to sexual maturity in the absence of other species.

In the present trials, a single species was unable to develop until the number of larvae used in exposure exceeded 400; actually, 6,000 larvae were used. The number of larvae used in exposures by Schwartz and Alicata was not reported.[3]

The present third trial (Table 3) deals with an experimental lungworm population as is evidenced by comparison of worm numbers. In the natural infections encountered in the second Wisconsin survey,[1] an average of only 34 *M. apri*, 23 *M. pudendotectus*, and 4 *M. salmi* were found.

An explanation of an association between species of lungworms is not inherent in data obtained to this time. Certainly no suggestion is made that the association is obligatory and, clearly, success of lungworm species in parasitizing a host is related to total number of larvae at exposure. It does appear, however, that an association of the species is characteristic of natural infections.

References

[1] Ewing, S. A., and Todd, A. C.: Metastrongylosis in the Field: Species and Sex Ratios of the Parasites, Preferential Location in Respiratory Apparatus of the Host, and Concomitant Lesions. Am. J. Vet. Res., 22, (May, 1961): 606–609.

[2] Goldsby, A., and Todd, A. C.: Helminth Infections in Wisconsin Market-Weight Swine. North Am. Vet., 38, (1957): 101–104.

[3] Schwartz, B., and Alicata, J. E.: Life History of Lungworms Parasitic in Swine. USDA Tech. Bull. 456, (1934): 41 pp.

[4] Todd, A. C.: Worm Parasites of Tennessee Chickens. Tenn. Agric. Exp. Sta. Bull. 205, (1948): 20 pp.

SUMMARIO IN INTERLINGUA

Association Inter Membros del Genere *Metastrongylus* Molin, 1861 (Nematoda : Metastrongylidae)

Esseva conducite tres experimentos pro demonstrar le existentia de un phenomeno de association inter *Metastrongylus apri* e *Metastrongylus pudendotectus*. Iste phenomeno ha previemente essite reportate per le autores comos un observation in populationes natural de vermes pulmonar. Tres differente magnitudes de inoculo—100, 400, e 6.000 larvas—esseva usate separatmente e in combination (i.e., *M. apri* sol, *M. pudendotectus* sol, e un combination de ambes). In expositiones a larvas de un sol specie, vermes non esseva retrovate si le inoculo non amontava a plus que 400 larvas, sed in expositiones combinate, jam 100 larvas sufficeva a producer infectiones. Le procentage del larvas del inoculo que esseva retrovate como vermes adulte post expositiones combinate esseva altissime in comparation illo post expositiones separate in le caso ubi le inoculo consisteva de 6.000 larvas. In plus, le maturation del vermes esseva accelerate le expositiones combinate. *M. pudendotectus* es minus capace que *M. apri* a successosemente parasitar porcos quando illo sol es presente. Isto es indicate per le micre procentage del inoculo retrovate e le relativemente reducite dimensiones del parasitos. Nulle base pro un explication de iste phenomeno esseva apparente ab iste experimentos. Le association del duo species non pare esser obligatori, sed illo es certo avantageose pro ambes.

Studies on a Skin Test for Leptospirosis

Donald C. Blenden, M.A., D.V.M.; Herbert S. Goldberg, Ph.D.; P. Kuppuswamy, B.V.Sc., M.S.

SUMMARY

A protein fraction of *Leptospira pomona*, S-91 was evaluated in a skin test for hypersensitivity to leptospires. This fraction was found to give a specific skin reaction when injected intracutaneously into guinea pigs sensitized with leptospires. The reaction, characterized by erythema, edema, and necrosis, appeared a few hours after injection of the fraction.

Evidence obtained indicated that the protein fraction is relatively stable and potentially useful in the detection of leptospirosis.

INTRODUCTION

Leptospirosis is an important disease affecting animals and man, and its control is closely linked with the finding of a simple, sensitive, diagnostic test suited for widespread application. Such a test is yet to be perfected. The possibility of evolving a skin test for leptospirosis was therefore investigated.

To date, a reliable skin test has not been employed with great success in any of the spirochetal diseases. Lutein tests employing suspensions of killed *Treponema pallidum* as skin test agents for diagnosis of syphilis were evolved by Noguchi,[6] but were extremely nonspecific. Jacobsthal [4] reported a skin test for leptospirosis which, however, was only preliminary and never confirmed. Stavitsky [9] reported hemorrhagic lesions at the site of injection four or five days after intradermal injection of *Leptospira icterohaemorrhagiae* into guinea pigs. It was not indicated whether this represented an allergic response or was due to toxic substances of spirochetal origin localized at the injection site by virtue of decreased capillary permeability. The author concluded that there were no further data on skin hypersensitivity in leptospirosis. Skin reactions were recorded in normal guinea pigs by Imamura *et al.*[3] after intradermal injections of an extract obtained from *L. icterohaemorrhagiae*. No reaction was observed, however, when a cellular extract of *Leptospira pomona* was injected intradermally into guinea pigs. Recently, it was observed in this laboratory [5] that marked erythema and swelling followed intradermal injection of a dialyzed preparation of sonicated cells of formalin-killed *L. pomona*, S-91. This reaction occurred in guinea pigs previously sensitized with *L. pomona* but did not occur in normal nonsensitized animals. An investigation of this reaction was consequently undertaken.

Schneider [7, 8] gave a comprehensive account of a fractionation procedure applied to leptospiral cells for a comparative yield of complement-fixing fractions from *L. icterohaemorrhagiae*, *Leptospira canicola*, and *Leptospira bataviae*. Leptospires were disrupted by chemical treatment and upon analysis yielded four nondialyzable, pentose-containing, serologically active fractions. Of the fractions, fraction III was predominantly protein (50 to 60%) and reactive in the complement-fixation test. It is this fraction which aroused our interest in a potential skin test antigen.

Materials and Methods

A fraction of *L. pomona*, S-91, designated III, was prepared according to the protocol described by Schneider.[8] The resulting fraction appeared as a white, particulate, protein residue. It was resuspended in phosphate-buffered saline solution (pH 7.1), standardized turbidimetrically* (50% transmission at 440 mμ) and injected intracutaneously on the backs of a series of nonsensitized and sensitized guinea pigs after the sites were clipped with disinfected clippers. The fractions were given in doses of 0.05 to 0.1 cc., which resulted in the appearance of a good-sized bleb (3 to 4 mm).

Seven- to 10-day-old, living, avirulent cultures of *L. pomona*, S-91, grown in 0.2% tryptose phosphate broth (Difco), to which 10% sheep serum was added, were used to sensitize guinea pigs. One milliliter of culture was given interaperitoneally,

Received for publication Feb. 8, 1961.

From the Schools of Medicine and Veterinary Medicine, University of Missouri, Columbia, Mo.

Supported in part by Grant-In-Aid E 2845, Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., and by funds from the Missouri Agricultural Experimental Station.

*Spectronic 20, manufactured by Bausch & Lomb, Rochester, N.Y.

and another 1.0 ml. was given seven days later by the same route. Nine guinea pigs (200 to 250 Gm.) were thus sensitized, and their serum titers were determined by the microscopic agglutination test, as described in detail in an earlier study.[2] If titers in some of the animals were not detectable at a minimum level of 1:50, a third 1.0-ml. injection of the culture, concentrated to 50% original volume, was given three weeks after the second injection. Serum titers determined after the third injection were always at 1:50 or higher.

Sensitized and nonsensitized control guinea pigs were kept in separate cages. After the injections of the fraction, observations were made every 30 minutes for the first few hours and thereafter twice daily. Four control and 9 sensitized guinea pigs were given injections of undiluted fraction III. In a second study, animals of a similar group were given injections of fraction III, diluted one hundredfold with sterile phosphate-buffered (pH 7.1) saline solution.

In order to investigate the relationship of the skin test fraction to cross specificity, a series of 30 guinea pigs was utilized. Ten each were sensitized with *L. pomona*, S-91, and *L. icterohaemorrhagiae* by intraperitoneal injection of viable cells suspended in saline soultion. The remaining 10 guinea pigs were used as nonsensitized controls. The sensitized animals developed agglutinin titers to the respective antigens and the control animals were serologically negative. All guinea pigs then were given intracutaneously 0.05 ml. of the undiluted fraction III suspension.

In a later study to determine the stability of the antigen, fraction III was heated to 60 C. for 30 minutes and tested, undiluted, as above, for skin test activity. In addition, a protein residue fraction, prepared in 1954, similar to Schneider's original preparation, was obtained (N. Rothstein, Walter Reed Army Institute of Research) and similarly tested.

Results and Discussion

The results of the first experiment, in which 4 control and 9 sensitized guinea

TABLE 1—Control and Sensitized Guinea Pigs Given Intradermal Injections of Fraction III Leptospira pomona Antigen

Guinea pig No.	Titer*	Reactions observed**				
		3 hr.	4 hr.	6 hr.	24 hr.	48 hr.
101	Negative controls	0	0	0	0	0
102	Negative controls	0	0	0	0	0
103	Negative controls	0	0	0	0	0
104	Negative controls	0	0	0	0	0
105	2,400	1	2	3 21 mm. diam.	2† 20 mm.	1 12 mm.
106	200	1	2	3 22 mm. diam.	1† 7 mm.	1 6 mm.
107	200	0	2	3 23 mm. diam.	1† 7 mm.	1 7 mm.
108	200	0	2	3 25 mm. diam.	2† 20 mm.	1 15 mm.
109	800	1	2	3† 22 mm. diam.	2† 17 mm.	1 13 mm.
111	50	1	2	3† 22 mm. diam.	2† 17 mm.	1 13 mm.
113	200	1	2	3 21 mm. diam.	3† 22 mm.	1 14 mm.
114	50	0	2	3 21 mm. diam.	3† 22 mm.	1 14 mm.
115	200	1	2	3 22 mm. diam.	2† 19 mm.	1 10 mm.

* Agglutinin titer to *Leptospira pomona* expressed as a reciprocal.

** 0 = no reaction; 1 = minimum reaction; 2 = intermediate reaction; 3 = maximum intensity reaction.

† Necrosis is reactive area; diameter of reactive area is shown in millimeters.

pigs were given fraction III intradermally, are shown (Table 1). In the sensitized guinea pigs, edema and erythema occurred within three hours after the injection. The edema was at a maximum for about 24 hours. At the end of 24 hours, ischemic areas with well-marginated, more or less circular, erythematous areas surrounding them had developed in all the sensitized animals. Bacteriologically sterile pus was noticed and, at 48 hours after injection, definite areas of necrosis were beginning to slough. Three to four days after the injection, ulcers remained, but by the sixth day the ulcers were healing. The maximum diameter of the skin lesion varied from 21 to 31 mm. The degree of skin test reaction appeared to have no correlation with the serum agglutinin titer of the animal.

Table 2 shows the results of the experiment in which diluted fraction III was used. In about four hours, erythema and edema were seen in all sensitized animals. In several animals there were slight areas of necrosis. The diameter of the skin lesions varied from 12 to 21 mm. as compared to 21 to 25 mm. seen in the first experiment in which the undiluted fraction III was used. The lesions in the sensitized animals followed the same course as in the first experiment; however, the lesions were less severe and healed more rapidly. In this experiment, the fact that a reaction was not seen in any of the normal controls and that milder but definite skin reactions occurred in all the sensitized animals indicated the effect of diluting the fraction.

All *L. icterohaemorrhagiae*-sensitized animals and *L. pomona*-sensitized animals reacted to fraction III by a central zone of ischemia which later became necrotic and ulcerated. The erythema was visible at three hours, and the erythematous reaction reached its maximal intensity at 24 hours, with a size varying from 21 to 30 mm. in diameter.

There was no indication that animals sensitized with *L. icterohaemorrhagiae* reacted less markedly than those sensitized with *L. pomona*. The control animals did not react.

The heated (60 C. for 30 minutes) preparation of fraction III and the fraction which had been stored at 4 C. for five years

TABLE 2—Control and Sensitized Guinea Pigs Given Intradermal Injections of 1:100 Dilution of Fraction III Leptospira pomona Antigen

Guinea pig No.	Titer*	Reactions observed**				
		3 hr.	4 hr.	6 hr.	24 hr.	48 hr.
116	Negative controls	0	0	0	0	0
117	Negative controls	0	0	0	0	0
118	Negative controls	0	0	0	0	0
119	Negative controls	0	0	0	0	0
120	200	2	2	3	Killed for histopathologic study	
121	50	2	2	3 21 mm.†	2 11 mm.	1 6 mm.
122	800	2	2	3 19 mm.	3 18 mm.	1 15 mm.
123	9,600	2	2	3 18 mm.	2 9 mm.	1 5 mm.
124	9,600	2	2	3 20 mm.	2 10 mm.	1 7 mm.
125	50	2	2	3 18 mm.	Killed for histopathologic study	
126	800	2	2	3 20 mm.	Killed for histopathologic study	
127	200	2	2	3 12 mm.	2 7 mm.	1 5 mm.
128	6,400	2	2	3 14 mm.	2 6 mm.	1 4 mm.

* Agglutinin titer to *Leptospira pomona* expressed as a reciprocal.

** 0 = no reaction; 1 = minimum reaction; 2 = intermediate reaction; 3 = maximum intensity reaction.

† Sizes refer to diameter of reactive area in millimeters.

at Walter Reed Army Institute of Research gave results identical to unheated, freshly prepared material. Thus, the skin test antigen is relatively stable to heat and cold.

Histopathology of Skin Test Lesions.—Three inoculation sites were excised 24 hours after inoculation for microscopic examination. A single section was cut through the center of the lesion and stained with hematoxylin and eosin.

The epidermis overlying the crest of the lesion had sloughed. The adjacent epithelium had undergone coagulation necrosis and was separated from the dermis by edematous fluid rich in neutrophils. Neutrophils had invaded the corneum at the margin of the lesion.

Neutrophils and a few scattered mononuclear cells infiltrated the entire dermis. Neutrophils were packed in the upper part of the dermis where collagen fibers and hair follicles were being destroyed. The deeper collagen fibers were separated by the cellular infiltration. The inflammatory reaction extended more widely in the subcutaneous tissue. Edema was more conspicuous at this site, but the leukocytic response was less intense and consisted of about equal numbers of neutrophils and mononuclear cells. There was mitotic activity in the latter cells. A few eosinophils were present. The leukocytes were concentrated around vessels and nerves in the subcutaneous tissue.

Several vessels in the subcutaneous tissue contained thrombi. Their walls were necrotic and invaded by neutrophils.

This reaction is quite similar to those previously reported [1] to be produced by tuberculin in tuberculosis-sensitized cattle. The fact that the reaction in leptospire-sensitized guinea pigs was characterized by intense neutrophilic infiltration would seem to be the major difference.

References

[1] Feldman, W. H., and Fitch, C. P.: Histologic Features of the Intradermic Reaction to Tuberculin in Cattle. Arch. Path., 22, (1936): 495–509.

[2] Higginbotham, H. B., Goldberg, H. S., and Logue, J. T.: Leptospiral Antibodies in Missouri Veterinarians. Vet. Med., 53, (1958): 581–585.

[3] Imamura, S., Iuribayashi, K., and Kameta, M.: Studies on Toxins of Pathogenic Leptospira. Jap. J. Micros., 1, (1957): 43.

[4] Jacobstal, E.: Die Agglomeration der spirochate de Weilschen Krankheitdurch Rekonvoleszentenserum. Deutsche med Wchnschr., 43((1917): 349–350.

[5] Kuppuswamy, P. B., Goldberg, H. S., and Blenden, D. C.: Studies on a Skin Test for Leptospirosis. Bact. Proc., (1960): 110–111.

[6] Noguchi, H.: A Cutaneous Reaction in Syphilis. J. Exptl. Med., 14, (1911): 557–568.

[7] Schneider, M. D., and McLaughlin, J.: Infrared Spectrophotometry of Cellular Constituents of Leptospires. J. Bact., 70, (1955): 85–91.

[8] Schneider, M. D.: Isolation and Chemical Composition of Complement Fixing Antigens from Leptospires. Proc. Soc. Exptl. Biol. & Med., 85, (1954): 32–37.

[9] Stavitsky, A. B.: Characteristics of Pathogenic Spirochetes and Spirochetosis with Special Reference to the Mechanisms of Host Resistance. Bact. Rev., 12, (1948): 203–255.

SUMMARIO IN INTERLINGUA

Studios in Re un Test Cutanee pro Leptospirosis

Un fraction proteinic de *Leptospira pomona*, S-91 esseva evaluate in un test cutanee pro hypersensibilitate contra leptospiras. Esseva trovate que iste fraction produce un specific reaction cutanee quando illo es injicite intracutaneemente in porcos de India sensibilisate con leptospiras. Le reaction, characterisate per erythema, edema, e necrosis, se manifestava pauc horas post le injection del fraction.

Le observationes indicava que le fraction de proteina es relativemente stabile e potentialmente utile in le detection de leptospirosis.

Direct Cultural Methods for the Isolation of Leptospires from Experimentally Infected Guinea Pigs

Robert W. Menges, D.V.M., M.P.H., and Mildred M. Galton, Sc.M.

SUMMARY

Direct culture of blood, urine, and kidney tissue for the isolation of leptospires from guinea pigs experimentally infected with *Leptospira pomona*, Wickard, was accomplished by: (1) inoculation of varying amounts of blood into Fletcher's medium, (2) culture of urine obtained from living animals by bladder tap, (3) culture of voided urine diluted in buffered NaCl solution, and (4) culture of urine and varying concentrations of kidney suspension obtained at necropsy. In addition, a variety of guinea pig tissues and body fluids were cultured at varying periods after infection to determine the location of leptospires in the body. Differences between inoculums of 1, 2, or 3 drops of blood per 5 ml. of medium were not observed. Leptospires were isolated from urine obtained by bladder tap from 91% of the samples cultured four to 15 weeks after inoculation.

Isolation of leptospires from kidney tissue of guinea pigs necropsied at least 20 days after inoculation was enhanced by culturing dilutions of 10.0, 1.0, and 0.1% of kidney suspension.

After the acute phase of illness, leptospires could not be isolated from tissues or body fluids except urine, and kidney and bladder tissues.

The greatest number of isolations were obtained with Fletcher's semisolid medium containing 10% pooled rabbit serum. Stuart's liquid medium and Chang's liquid and semisolid mediums were less satisfactory for initial isolation.

Received for publication Dec. 22, 1960.

From the Epidemiology Branch, Veterinary Public Health Laboratory Unit, Communicable Disease Center, Public Health Service, United States Department of Health, Education, and Welfare, Atlanta, Ga.

INTRODUCTION

It is generally agreed that isolation of leptospires by direct culture of blood, urine, or kidney tissue is the most desirable method of laboratory diagnosis.[4, 7] However, there is considerable variation as to the recommended proportion of inoculum to culture medium, the method of collection and preparation of body fluids and tissues,[8] and the period that may safely elapse between collection of samples and inoculation of the culture medium. The results of comparative studies for the isolation of *Leptospira pomona* by direct culture from blood, urine, and tissues of infected guinea pigs are presented.

Materials and Methods

In August, 1955, guinea pigs infected with *L. pomona*, Wickard, were received from Dr. E. V. Morse.* The guinea pigs were necropsied and kidney suspensions used to start two passage lines. There were 50 passages made with one line and 25 with the other. Blood, urine, and kidney from the guinea pigs in these 75 passages were used in an effort to group and evaluate techniques for isolation of *L. pomona*.

At each passage, the guinea pigs were anesthetized with ether and blood was obtained from the heart for serologic tests. The guinea pigs were then killed, and varying concentrations (as indicated below) of urine and kidney tissue were cultured. The kidney suspensions from all of the guinea pigs at each passage were pooled, and 1.0 to 2.0 ml. was inoculated intraperitoneally into 3 weanling (4-week-old) English short-haired guinea pigs weighing approximately 200 Gm. In addition, one drop of the kidney suspension was dropped into each eye of the guinea pigs. Unless otherwise stated, sterile, dry, 2-ml. syringes with 1-inch, 20-gauge needles were used.

During the first passages, rectal temperatures taken daily for two weeks generally increased to 104 F. or greater four to eight days after inoculation, so temperatures were taken routinely during this period only. When the fever developed, the

* *Leptospira pomona*, Wickard, was isolated by Dr. E. V. Morse in Wisconsin in 1953 by inoculating bovine urine intraperitoneally into guinea pigs. The strain was passed in guinea pigs every three to four weeks. For passage, a 10% suspension of kidney was prepared at necropsy and 2.0 ml. was inoculated intraperitoneally.

guinea pigs were anesthetized with ether, blood was obtained from the heart, and the whole blood was cultured.

Blood.—The thoracic area was swabbed with 70% alcohol, and blood was obtained with a sterile, dry, 10-ml. syringe with a 2½-inch, 20-gauge needle. Three drops of whole blood were inoculated immediately into each of four to six tubes containing 5-ml. amounts of Fletcher's medium.[3] To determine the desired proportion of inoculum to medium, 1, 2, and 3 drops of blood from each of 43 animals was inoculated into each of two tubes of Fletcher's medium.

Urine.—Urine was obtained from 19 infected guinea pigs by the bladder tap technique.[5] The guinea pig was held on its back with the left hand grasping the right hind leg. The abdomen was swabbed with 70% alcohol, and the bladder tapped. One to 3 drops of urine were inoculated into each of four tubes of medium. If the bladders were empty, 10 ml. of physiologic saline solution was injected intraperitoneally and the bladder tapped 15 to 30 minutes later.

Urine voided from 15 guinea pigs was obtained for direct culture by a dilution technique previously described.[6] Thirty-nine samples were obtained six days to 17 weeks after inoculation.

Urine from 3 guinea pigs infected with *L. pomona*, Wickard, and necropsied on the 28th day postinoculation was used to determine the period that *L. pomona* would survive in the urine after death of the animal. Infection had been proved by isolation of leptospires from the blood. In the first experiment, urine was collected from two guinea pigs by the bladder tap method. The urine was obtained seven minutes after death of the guinea pig, and three drops was inoculated immediately into each of three tubes of Fletcher's medium. The urine was held in the syringes at room temperature. Urine from the first guinea pig was cultured only after one hour but that from the second guinea pig was cultured at hourly intervals for six hours. The bladder of the third guinea pig was tapped at 15 minutes, two hours and 15 minutes, and four hours after death. At the end of four hours, the bladder was empty so 0.5 ml. of Fletcher's medium was injected into it and a portion was withdrawn at six and eight hours.

Kidney.—Kidney cultured at necropsy was obtained from the guinea pigs as soon as possible after death. In addition, urine samples obtained by bladder tap at necropsy were cultured for comparison. Prior to necropsy, the animals were soaked in 10% liquor cresolis for approximately 15 minutes. Sterile instruments were used to remove the skin, then with another set of sterile instruments, the muscle and peritoneum were removed and the viscera exposed. Urine was collected by the bladder tap technique and cultured as described.

Both kidneys were removed and weighed in a sterile petri dish. They were then placed in a sterile mortar, sterile sand was added, and the mixture ground thoroughly. Sterile buffered saline solution (1,840.0 ml. of 0.85% NaCl plus 160.0 ml. of Sorensen's buffer) was added to make a 10.0% tissue suspension (9.0 ml. of buffered saline solution per 1 Gm. of tissue). After mixing well into solution, the supernatant fluid was poured into a sterile glass tube and 1.0 ml. of the suspension was withdrawn. Three drops of the suspension was inoculated immediately into each of four to six tubes of the medium.

Different concentrations of kidney suspension were also prepared as follows: 50.0%, 4 Gm. of kidney plus 4.0 ml. of buffered saline solution; 33.0%, 4 Gm. of kidney plus 8.0 ml. of buffered saline solution; 10.0%, 4 Gm. of kidney plus 36.0 ml. of buffered saline solution; 1.0%, 0.1 ml. of the 10.0% suspension plus 0.9 ml. of buffered saline solution; and 0.1%, 0.1 ml. of the 1.0% suspension plus 0.9 ml. of buffered saline solution. Each suspension was then cultured by inoculation of three drops per 5.0 ml. of medium.

Eight *L. pomona*-infected guinea pigs were necropsied at four, ten, 20, 30, 40, 52, 70, and 137 days postinoculation, and 3 drops of a 10% suspension of each tissue or 3 drops of the fluid samples was inoculated into each of three to six tubes of medium. Fluids and tissues cultured included: aqueous and vitreous humor, urine, bladder, kidneys, adrenals, liver, bile, spleen, pancreas, mesenteric lymph nodes, submaxillary lymph nodes, salivary glands, uterus, ovaries, testicles, heart, lungs, diaphragm, thigh muscles, cerebrospinal fluid, spinal cord, and brain.

Fletcher's semisolid,[4] Stuart's liquid,[9] and Chang's liquid and semisolid mediums[1] were compared for isolation of leptospires. Sterile hemolyzed rabbit serum was added to all mediums to provide a final 10% concentration. The mediums were dispensed in 5.0-ml. amounts into screw-capped test tubes (16 mm. by 125 mm.) and then tested for sterility by incubation at 37 C. for 24 hours. The ability to support growth of leptospires before use was tested by inoculation of 0.1-ml. amounts of actively growing stock leptospiral serotypes into the mediums.

The microscopic agglutination test[2] with formalinized *L. pomona*, S91, antigen was used with serum from 55 of the infected guinea pigs.

Results

Of 167 *L. pomona*-infected guinea pigs, 155 (99%) had a rectal temperature of 103 to 104 F., or above, between the fourth and eighth days after inoculation. Leptospires were isolated from the blood of 115

TABLE 1—Blood Cultures from 43 Guinea Pigs Inoculated with Leptospira pomona; Varying Amounts of Inoculum Used

Blood specimens	No. of drops			Total specimens
	1	2	3	
No. cultured	43	38	43	43
Leptospires isolated	28	23	30	34
% positive	65.2	60.5	69.8	79.1

TABLE 2—Summary of the Culture Findings on 49 Urine Specimens Obtained by Bladder Tap 1 to 15 Weeks after Inoculation from Guinea Pigs Infected with Leptospira pomona, Wickard

	Weeks after inoculation													
	1	2	3	4	5	6	7	9	11	12	13	14	15	Total
N	4	11	12	7	3	2	1	2	2	1	1	1	2	49
+	1	7	6	6	3	2	1	2	2	1	0	1	2	34
%	25	64	50	86	100	100	100	100	100	100	0	100	100	69
ND	4	11	12	7	3	2	1	2	2	1	1	1	2	49
+D	0	1	4	1	0	1	0	0	0	0	0	0	0	7
%	0	9	33	14	0	50	0	0	0	0	0	0	0	14

N = Number of urine specimens. None of the specimens were contaminated; + = Leptospires isolated; +D = Leptospires observed by darkfield examination; ND = Number of darkfield examinations; % = Per cent positive.

(78%) of 148 guinea pigs between the fourth and tenth days. With an inoculum of 1 drop of blood, 28 (65.2%) of 43 samples were positive; with 2 drops, 23 (60.5%) of 38 samples were positive, and with 3 drops, 30 (69.8%) of 43 samples were positive (Table 1).

A summary of findings on culture of 49 urine specimens obtained by bladder tap from 19 infected guinea pigs from one to 15 weeks after inoculation is given (Table 2). Leptospires were isolated from 34 (69%) of the 49 specimens and none were contaminated. Leptospires were found in only seven (14%) of the urine samples by direct darkground examinations.

Direct culture of voided urine samples by the dilution technique also proved to be a satisfactory method for recovery of leptospires. Isolations were made from a total of 23 (59%) of the 39 urine samples cultured. Pure cultures were found in one or more tubes in 21 (91%) of the 23 positive specimens. The percentage of positive increased from six (15%) in tubes inoculated with undiluted urine to 13 (42%) in those inoculated with the third dilution and were found at this level also in the fifth dilution as shown (Table 3).

In an experiment to determine how long leptospires would survive in guinea pig urine, isolations were obtained from the urine of 1 infected guinea pig when cultured one hour after collection and from the second animal up to six hours after death. These samples were held in the syringe at room temperature. At necropsy of another infected guinea pig, urine was allowed to remain in the bladder and obtained by bladder tap at intervals up to four hours for culture. Leptospires were isolated from all samples cultured. As the bladder was empty at this time, Fletcher's medium was injected and the bladder tapped again at six to eight hours, and leptospires recovered. Thus, it appears that *L. pomona* will remain viable in guinea pig urine at room temperature for at least six hours and in the bladder of a dead guinea pig for four to eight hours.

Kidney suspensions were cultured from 104 *L. pomona*-infected guinea pigs at necropsy 20 to 42 days after inoculation and leptospires were isolated from 74 (71%). At the same time, urine was cultured from 74 of these guinea pigs for comparison. Leptospires were isolated from 53 (72%) of the kidney cultures and 47 (64%) of the urine cultures. Positive cultures from the urine only were obtained in 14 (19%) of the samples and from the kidney only in eight (11%) as shown (Table 4). While more isolations were made from the kidney cultures, leptospires would have been missed in 6 (8%) of these guinea pigs if the urine had not been cultured also.

Five different dilutions of kidney suspension were tried. A 50.0% kidney suspension from 8 animals was cultured, with only two samples (25%) positive; de-

TABLE 3—Summary of Findings on 39 Voided Urine Specimens from 15 Guinea Pigs Experimentally Infected with Leptospira pomona, Wickard

	No. cultured	Positive		Contaminated	
		No.	%	No.	%
Undiluted	39	6	15	33	85
Dilution 1	37	6	16	26	70
Dilution 2	36	14	39	11	31
Dilution 3	31	13	42	8	26
Dilution 4	31	7	23	9	29
Dilution 5	31	13	42	5	16

TABLE 4—Comparative Findings on Culture of Urine and Kidney from 74 Guinea Pigs Infected with Leptospira pomona, Wickard

Specimen	No. cultured	Positive	
		No.	%
Urine	74	47	64
Kidney	74	53	72
Urine only	74	14	19
Kidney only	74	8	11
Totals	74	67	90

creasing the concentration, a 33.0% suspension from 20 animals was cultured and eight samples (40%) were positive. For the majority of cultures, a 10.0% suspension was used. Of 76 samples cultured, six were grossly contaminated but leptospires were isolated from 59 (81%) of the remaining 70 cultures. In addition, 1.0% kidney suspensions were prepared from 21 of these guinea pigs and 0.1% suspensions from 11 for comparison. The number of isolations increased as the kidney suspensions became more dilute, with 65%, 81%, and 90% isolations being achieved with the 10.0%, 1.0%, and 0.1% concentrations (Table 5).

Another 10 experimentally infected guinea pigs were necropsied on the sixth day after inoculation and 10.0%, 1.0%, and 0.1% kidney suspensions were cultured. Five of these animals were infected with *L. pomona*, Wickard, and 5 with *L. icterohaemorrhagiae*, Wijnberg. Leptospires were isolated from eight (80%) of the 10% suspensions, seven (70%) of the 1.0% suspensions, and five (50%) of the 0.1% suspensions (Table 5). Five hamsters infected with *L. canicola* were necropsied on the sixth day after inoculation and different concentrations of kidney tissue were cultured for comparison. Of these, 4 (80%) of the hamsters were found to be positive when 10.0% kidney suspension was used, 1 (20%) with a 1.0% suspension and none with 0.1% suspension. It appears from the comparative kidney cultures made between 20 and 42 days after inoculation that the more dilute inoculum of 0.1% should be used, but if kidney cultures are obtained as early as the sixth day after infection, the more concentrated inoculum of kidney suspension (10.0%) is indicated.

In the experiment to determine the location of leptospires in the body at different stages of infection, numerous tissues and body fluids were cultured four to 137 days after inoculation. All guinea pigs studied were positive by blood culture (Table 6). Leptospires were isolated from the blood, kidney and liver tissues, and spinal fluid of the guinea pig necropsied on the fourth day postinoculation. From guinea pigs necropsied after this period, isolations were obtained only from the kidney tissue, urine, or bladder. Thus, it appears that if leptospires are present in organs other than the kidney and bladder after the acute phase of infection, they are in extremely small numbers.

Aqueous and vitreous humor were cultured from 30 *L. pomona*-infected guinea pigs proven positive by blood culture. In 16 of these guinea pigs, inoculated intraperitoneally with 2.0 ml. of a 10% kidney suspension, all of the eye fluid cultures were negative. In the remaining 14 guinea pigs, a drop of the 10% kidney suspension was placed into each eye and 2.0 ml. was inoculated intraperitoneally. Leptospires were isolated from the ocular fluid of 2 (14%) of these guinea pigs when necropsied on the 28th day postinoculation. Scarring of the cornea was observed in one of these positive animals. Infection could not be detected by study of blood, urine, kid-

TABLE 5—Comparative Findings on Culture of Various Dilutions of Kidney Suspensions from Experimentally Infected Guinea Pigs and Hamsters

Infecting strain	Test animal	Necropsied	% kidney suspension	No. cultured	Positive	
					No.	%
L. pomona Wickard	guinea pigs	20–42nd day	10.0	21	13	65.0
			1.0	21	17	81.0
			0.1	11	10	90.0
L. pomona Wickard [5]	guinea pigs	6th day	10.0	10	8	80.0
L. icterohaemorrhagiae [5]			1.0	10	7	70.0
			0.1	10	5	50.0
L. canicola	hamsters	6th day	10.0	5	4	80.0
			1.0	5	1	20.0
			0.1	5	0	0

TABLE 6—Summary of Findings on Culture of Different Tissues and Fluids from 8 Guinea Pigs Infected with Leptospira pomona, Wickard

Guinea pig No.	Highest temperature (°F.)	Blood culture: Day	Blood culture: Results	Day cultured	Eye fluid	Urine	Bladder	Kidney	Adrenals	Liver	Bile	Spleen	Pancreas	Mesenteric lymph nodes	Submaxillary lymph nodes	Salivary glands	Uterus	Ovaries	Testicles	Heart	Lung	Diaphragm	Thigh muscles	Cerebrospinal fluid	Spinal	Brain
		Specimen cultured																								
1	104.8	4	+	4				+		+														+		
2	105.4	4	+	10				C				C									C					
3	105.6	5	+	20			+	+																		
4	105.2	6	+	30		+		+	C	C		C		C						C						C
5	105.4	4	+	40		+	+	+	C	C		C						C			C					
6	106.2	4	+	52		+	C	+																		
7	106.4	5	+	70		+		+													C					
8	105.2	5	+	137	C												C									

C = contaminated.

TABLE 7—Findings on Four Leptospiral Mediums Inoculated with Blood, Urine, or Kidney Tissue Samples from Guinea Pigs Experimentally Inoculated with Leptospira pomona

Medium	Specimens cultured: No.	Positive: No.	Positive: %
Fletcher's semisolid	195	93	48
Stuart's liquid	165	11	7
Chang's semisolid	63	3	5
Chang's liquid	83	1	1
Totals	287	103	36

ney tissue, or ocular fluid in 3 guinea pigs inoculated by the conjunctival route only.

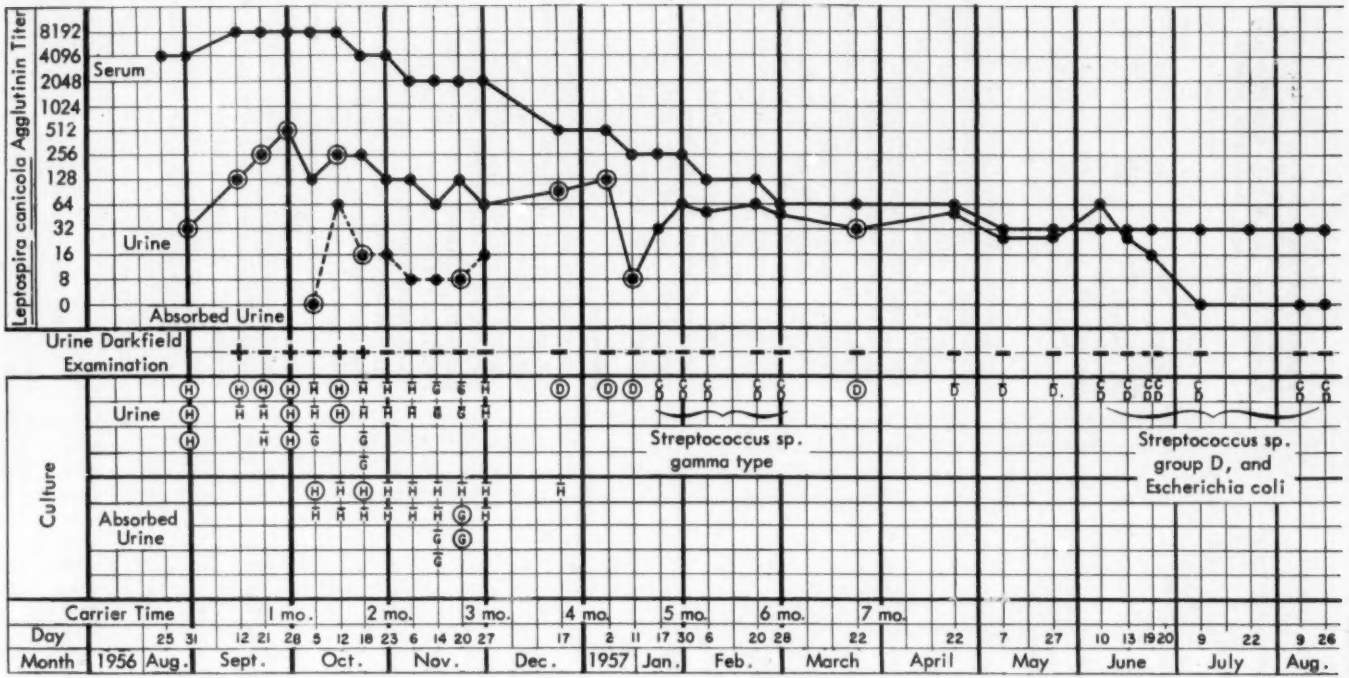
Stuart's liquid medium and both the liquid and semisolid mediums of Chang were used comparatively with Fletcher's semisolid medium. By far the greatest number of isolations were obtained on Fletcher's medium (Table 7).

Serum samples obtained from 55 guinea pigs between 20 and 42 days postinoculation were tested by the microscopic agglutination test with formalinized *L. pomona* antigen. Antibodies against *L. pomona* were detected in all samples. Titers ranged from 1:32 to 1:1,024 with 43 (78%) samples between 1:64 and 1:256.

Discussion

For many years the accepted procedure for isolation of leptospires from contaminated specimens has been an indirect method involving inoculation of laboratory animals,[3] subsequent periodic cardiac puncture, culture of the blood, and, finally, culture of the kidneys. This method has many disadvantages, as previously pointed out.[5] It has been shown in recent studies [6] that leptospires may be isolated with relative ease from voided urine specimens, which are usually contaminated, by direct culture if a dilution technique is used. Further evidence of the successful application of direct culture techniques has been presented. It seems apparent, therefore, that one or more of these direct culture methods should always be used when isolation of leptospires is attempted and that animal inoculation would be indicated only as an ancillary method in unusual circumstances.

While the guinea pig experiments were being done, limited studies were conducted on naturally occurring cases of canine leptospirosis to determine whether the laboratory experiments were of real value for



O - Lept. canicola isolated
\+ - Darkfield examination positive
— - Negative
C - All culture tubes contaminated
H - Hamster
G - Gerbil
D - Direct culture after bladder tapping

Fig. 1—Serologic and cultural studies on a canine Leptospira canicola carrier.

routine application. In the early phases of the work, a male crossbred Pointer was obtained, and urine specimens were cultured for four months by the animal inoculation method. The urine was positive for eight consecutive weeks by this method, and then became negative on repeated cultures during the following two months. At that time, the dog was thought to have stopped shedding leptospires in the urine.

Later, as the work progressed, the bladder tap technique was developed, and it was tried on another canine *L. canicola* carrier. Urine specimens from this dog became negative by the standard animal inoculation method by the second month. By absorbing the urine, using the method described by Stuart,[10] and then inoculating laboratory animals, the urine was found to be positive almost another month. By the third month, however, the urine was negative by the animal inoculation method. At that time, the serum titer was still 1:2,048 (Fig. 1). Urine specimens after the third month were obtained by the bladder tap technique and inoculated directly into a culture medium. This dog was proved to be a shedder of leptospires for an additional four months, a total of seven months (Fig. 1). These studies of naturally occurring cases gave definite evidence of the value of the experimental procedures developed.

Conclusions

Under the conditions of these experiments, the following conclusions were drawn:

1) For direct culture of blood, 1 to 3 drops should be inoculated into four to six tubes containing 5 ml. of medium.

2) Dilutions of at least 10.0, 1.0, and 0.1% kidney suspension should be cultured for isolation of leptospires from kidney tissue.

3) Voided urine is satisfactory for the isolation of leptospires if it is diluted serially in buffered saline solution before culture.

4) Both kidney tissue and urine should be cultured at necropsy.

5) Leptospires will survive for at least six hours after death in guinea pig urine held at room temperature.

References

[1] Chang, S. L.: Studies on *Leptospira icterohaemorrhagiae*. J. Infect. Dis., 81, (1947): 28–34.

[2] Fischer, G. W., Powers, D. K., and Galton, M. M.: Studies of Technics for the Isolation of Leptospires. I. The Suitability of 1- to 2-Day Old Baby Chicks as Compared to Hamsters for the Isolation of Various Leptospiral Serotypes. J. Infect. Dis., 103, (1958): 150–156.

[3] Galton, M. M., Powers, D. K., McKeever, S., and Gorman, G. W.: Identification of Two Leptospiral Serotypes New to the United States. Public Health Rep., 72, (1957): 431–435.

[4] Gochenour, W. S., Yager, R. H., Wetmore, P. W., and Hightower, J. A.: Laboratory Diagnosis of Leptospires. Am. J. Public Health, 43, (1953): 403–414.

[5] Menges, R. W., Galton, M. M., and Hall, A. C.: Diagnosis of Leptospirosis from Urine Specimens by Direct Culture Following Bladder Tapping. J.A.V.M.A., 132, (1958): 58–60.

[6] Menges, R. W., Rosenquist, B. D., and Galton, M. M.: A Method for the Isolation of Leptospires from Voided Urine by Direct Culture. J.A.V.M.A., 137, (1960): 313–316.

[7] Ross, C. J.: Blood Culture in the Diagnosis of Leptospirosis in North Queensland. Med. J. Australia, 2, (1956): 319–322.

[8] Rudge, J. M.: Observations on the Efficiency of Animal Inoculation for Isolating Leptospires from Kidney Tissue. New Zealand Vet. J., (1958): 15–16.

[9] Stuart, R. D.: The Preparation and Use of a Simple Culture Medium for Leptospirae. J. Path. Bact., 58, (1946): 343–349.

[10] Stuart, R. D.: The Importance of Urinary Antibodies in the Diagnosis of Leptospirosis. Canad. J. Microbiol., 2, (1956): 288–297.

SUMMARIO IN INTERLINGUA

Directe Methodos Cultural pro le Isolation de Leptospiras ab Experimentalmente Inficite Porcos de India

Un culturation directe de sanguine, urina, e tissu renal pro le isolation de leptospiras ab porcos de India experimentalmente inficite con *Leptospira pomona*, Wickard, esseva complite per (1) le inoculation de varie quantitates de sanguine in medio de Fletcher, (2) le culturation de urina obtenite ab animales vive per punctiuras de vesica, (3) le culturation de vacuate urina diluite in un tamponate solution de NaCl, e (4) le culturation de urina e suspensiones renal de varie concentrationes obtenite al necropsia. In plus, varie tissus e liquidos corporee de porco de India esseva culturate a varie periodos post le infection pro determinar le location de leptospiras in le corpore. Esseva observate nulle differentias inter inoculos de 1, 2, o 3 guttas de sanguine per ml de medio. Leptospiras esseva isolate ab urina obtenite

per punctura vesical in 91% del specimens culturate inter quatro e 15 septimanas post le inoculation.

Le isolation de leptospiras ab tissu renal de porco de India obtenite per necropsias al minus 20 dies post le inoculation esseva promovite per le uso, in le culturation, de suspensiones renal de dilutiones de 10,0, 1,0, e 0,1%.

Post le acute phase del morbo, leptospiras non poteva esser isolate ab ulle tissu o liquido corporee, con le exception de urina e de tissu renal e vesical.

Le plus grande numero de isolationes esseva obtenite con un medio semisolide de Fletcher que contineva 10% de sero de conilio de origine collective. Le medio liquide de Stuart e le medios liquide e semisolide de Chang esseva minus satisfactori pro le isolation initial.

Estimation of Cardiac Output by the Direct Fick Technique in Domestic Animals, with Observations on a Case of Traumatic Pericarditis

C. M. Stowe, V.M.D., Ph.D., and A.L. Good, V.M.D., Ph.D.

SUMMARY

The cardiac output of several anesthetized animals, presumably representative of four species of the larger domestic animals, was obtained by the direct Fick oxygen technique. In general, the cardiac output of these animals was in the range of 70 to 150 ml./kg./min., while cardiac indexes ranged from approximately 2 to 9, with most of the values around 4 to 6. In a heifer affected with traumatic pericarditis, a relatively low cardiac output and stroke index was found, along with elevation of the jugular and right ventricular pressures.

INTRODUCTION

The cardiac output is defined as the volume of blood ejected by the heart per minute. This study was undertaken in an effort to gather information of a comparative physiologic nature on this important parameter of cardiovascular function in several species of domestic farm animals.

Materials and Methods

All the animals used in this study were anesthetized to plane 1 or plane 2 of surgical anesthesia. The sheep, goat, and all the calves except one were given pentobarbital sodium intravenously at a total dose of 15 to 25 mg./kg. The cows and calf 2 received 6% chloral hydrate solution intravenously at an initial dose of 130 to 170 mg./kg. The swine were anesthetized with 300 mg./kg. doses of chloral hydrate given as a 5% solution intraperitoneally. Surgical anesthesia was atained in about 20 minutes. The horses were restrained initially with a rapid intravenous injection of a mixture containing 0.12 mg./kg. of succinylcholine chloride and approximately 650 mg. of pentobarbital sodium. These two agents were mixed immediately before use. Upon collapse, 8% chloral hydrate solution was administered intravenously until anesthesia supervened. This usually required about 130 mg./kg.

After the animals were anesthetized, a tracheal cannula of appropriate size was inserted. One carotid artery was cannulated. In addition, a catheter was passed to the right ventricle via a 12-gauge needle inserted into the jugular vein. The location of the catheter tip was monitored by means of a mercury manometer.

The tracheal cannula was connected by a short length of tubing to a Benedict-Roth metabolism apparatus. A period of five to ten minutes of oxygen breathing was permitted in each case to ensure that steady state conditions prevailed, a necessary requirement for valid Fick determinations. In the case of the larger animals, it was necessary to recharge the spirometer with oxygen frequently. Arterial and mixed venous blood samples were drawn slowly under anaerobic conditions, and their oxygen contents determined by the Van Slyke manometric method. After measuring the oxygen consumption (corrected to N.T.P.), the cardiac output was calculated. In each animal, two or more sets of samples were taken and the results averaged. The cardiac index was calculated from the cardiac output and the surface area, as obtained from Meeh's formula: $A = k W^{2/3}$, where $k = 0.105$ for calves, 0.121 for sheep, and 0.087 for pigs.[11]

Results

The data on oxygen consumption, A.V. oxygen difference, cardiac output, and cardiac index obtained from the various animals studied are presented (Tables 1–4).

During the course of these experiments, a 900-lb. (409-kg.) grade Holstein-Friesian heifer, afflicted with traumatic pericarditis, became available for study. The procedures

Received for publication Sept. 1, 1960.

From the Division of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, University of Minnesota.

Part of the material in this article was presented at the Research Section of the American Veterinary Medical Association Convention, Kansas City, Mo., Aug. 23–27, 1959.

This work was supported in part by financial assistance from the North Central Regional Research Committee (NC-27).

Minnesota Agricultural Experiment Station Science Journal Series Article No. 4470.

The authors thank Miss Angelika Jegers for her assistance with the Van Slyke determinations, and Mr. Fred Borgwardt for his assistance with the preparation of animals.

TABLE 1—Cardiac Output in Anesthetized Calves

Calf No.	Body weight (kg.)	O_2 consumption (ml./min.)	A.V. oxygen difference (ml./liter)	Cardiac output (liter/min.)	Cardiac output (ml./kg./min.)	Cardiac index (liter/M²/min.)
1	70	358	55.5	6.4	91	3.6
2	107	425	34.0	14.5	135	6.1
3	34	223	52.6	4.2	123	3.9
4	73	430	46.5	9.4	129	5.1
5	98	591	57.5	10.3	105	4.6
6	69	365	57.5	6.4	93	3.6
7	80	451	54.2	8.3	104	4.3
8	75	307	28.9	10.9	145	9.2
9	67	358	52.4	6.8	102	4.0
10	45	271	40.1	6.8	151	5.1
11	45	275	42.2	6.5	144	4.9
Mean	69	369	47.5	8.2	120	5.0

TABLE 2—Cardiac Output in Anesthetized Swine

Pig No.	Body weight (kg.)	O_2 consumption (ml./min.)	A.V. oxygen difference (ml./liter)	Cardiac output (liter/min.)	Cardiac output (ml./kg./min.)	Cardiac index (liter/M²/min.)
1	25	212	39.7	5.3	214	7.2
2	50	420	74.8	5.6	112	4.8
3	36	202	62.8	3.2	89	3.4
4	19	143	58.0	2.2	117	3.6
5	30	249	36.6	6.8	225	8.1
6	28	172	40.8	4.2	150	5.3
7	34	239	60.8	3.9	116	4.3
Mean	32	234	53.4	4.5	146	5.2

TABLE 3—Cardiac Output in Anesthetized Ewes

Ewe No.	Body weight (kg.)	O_2 consumption (ml./min.)	A.V. oxygen difference (ml./liter)	Cardiac output (liter/min.)	Cardiac output (ml./kg./min.)	Cardiac index (liter/M²/min.)
1	50	235	38.1	6.2	124	3.8
2	61	296	50.5	5.9	97	3.1
3	39	191	69.4	2.8	72	2.0
4	49	208	46.2	4.5	92	2.8
5	51	379	54.3	7.0	137	4.2
Mean	50	262	51.7	5.3	104	3.2

TABLE 4—Cardiac Output in 3 Anesthetized Horses, a Cow, and a Goat

Species	Body weight (kg.)	O_2 consumption (ml./min.)	A.V. oxygen difference (ml./liter)	Cardiac output (liter/min.)	Cardiac output (ml./kg./min.)	Cardiac index (liter/M²/min.)
Horse	280	1,694	65.2	26.0	93	6.1
Horse	143	693	67.9	10.2	71	3.7
Horse	314	2,110	50.6	41.7	133	9.0
Goat	41	175	42.7	4.1	101	2.9
Cow	318	820	22.8	35.9	113	7.3

TABLE 5—Cardiovascular Observations in a Heifer (409 kg.) with Traumatic Pericarditis

Jugular pressure	50 mm. Hg
Right ventricular pressure	70 mm. Hg
Oxygen consumption	1007 ml./min.
Cardiac output	18.9 liters/min. (46 ml./kg./min.)
Heart rate (obtained by EKG)	160 per min.
Stroke volume	118 ml.
Cardiac index	2.4 liter/M²/min.

described for the other animals were employed and the findings are reported (Table 5).

At necropsy, this heifer was found to have an enlarged pericardial sac which contained a great volume of seropurulent exudate, containing clumps of fibrin. The pericardium was 25 cm. in diameter, and the wall of the organ was 1 to 2 cm. in

thickness. There was evidence of myocardial deterioration.*

Discussion

Since all of these animals were anesthetized to plane 1 or 2 of surgical anesthesia before the rate of oxygen consumption and blood oxygen content were determined, consideration must be given to the effect of general anesthetics on cardiovascular and, in the case of direct Fick determinations, respiratory function as well. Greisheimer *et al.*[8] reported some increase in cardiac output in dogs anesthetized with thiopental sodium. Stowe and Good [15] studied cardiac output in both anesthetized and non-anesthetized calves and reported that there was no marked or regular difference between the two (118 ml./kg./min., and 123 ml./kg./min., respectively). On the other hand, the authors observed in the current experiments that the cardiac output of calves declined after one to six hours of pentobarbital anesthesia.

Hemingway [9] recently summarized much of the work that has been done on anesthetized dogs and found that, in general, the cardiac output declined after the inception of anesthesia. In his own experiments, he also noted decreased cardiac output with pentobarbital anesthesia; his minimum values, for example, were obtained 15 to 35 minutes after administration of the drug. Since barbiturates depress oxygen consumption, it would seem logical to expect a fall in cardiac output, as calculated from the direct Fick principle, if one assumes a constant arterial-venous oxygen difference, or even an A.V. oxygen difference that decreases at a rate less than the rate of decrease in oxygen consumption.

One problem that must be recognized in estimating cardiac output by this method is the lack of precision with the methods that are available. Variations as high as 15%, and sometimes greater, are not uncommon in a series of cardiac output estimates made at short time intervals. One possible explanation for this lies in the fact that oxygen consumption records obtained from the Benedict-Roth metabolism apparatus may be in error by as much as 10%.[7]

The results obtained with these experiments were similar to those reported elsewhere. Zuntz and Hagemann [17] found that cardiac output values in nonanesthetized horses averaged 25% lower than those reported in this study; Barcroft *et al.*,[1] in studies with nonanesthetized goats, obtained cardiac output values which were quite similar. More recently, Schambye (sheep),[14] Doyle *et al.* (cows),[5] Fisher and Dalton (cows),[6] and Stowe and Good (calves and sheep) [15] reported cardiac output values in the same general range as those for the animals in this study.

The low cardiac output found in the heifer with traumatic pericarditis seems reasonable, particularly in view of the elevated pressure in the jugular vein and the right ventricle. The stroke volume was substantially below the figure of 637 ml. that was found by Doyle *et al.*[5] in dairy cows of comparable weight. Presumably, the constriction due to the distended pericardial sac impeded the return of blood to the heart, and limited the diastolic filling of the ventricles. Abnormally low cardiac output values and greatly elevated venous pressures have been observed in human beings with constrictive pericarditis.

Burwell and Strayhorn [4] and Burwell and Flickinger [3] reported that elevated venous pressures, reduced cardiac outputs, and reduced cardiac indexes in constrictive pericarditis improved, following resection of the pericardium. Similar findings also were reported by Lyons and Burwell,[12] along with the additional observation that tachycardia, induced by intravenous atropine sulfate, was accompanied by an increase in the cardiac output and a fall in the venous pressure. Wilson *et al.*[16] found cardiac indexes which were about 50 to 60% of normal, and increased pressure in the right side of the heart and the vena cava. There also have been some interesting studies on dogs in which experimental constrictive pericarditis was produced by placing excess saline solution or various irritant materials into the pericardial sac. Such animals were of particular value in following the cardiovascular changes induced by such procedures.[2,10,13] In general, reduction of the cardiac index and elevated venous pressures were reported. These reports suggest that similar studies might well be fruitful in order to better understand traumatic pericarditis in cattle.

* The authors thank Dr. N. O. Nielsen of the Division of Veterinary Pathology and Parasitology for conducting the pathologic examination.

References

[1] Barcroft, J., Boycott, A. E., Dunn, J. S., and Peters, R. A.: Observation on Respiration and Circulation in the Goat. Quart. J. Med., 13, (1919): 35–45.

[2] Boucek, R. J., Grindlay, J. H., and Burchell, H. B.: Experimental Constrictive Pericarditis: Analysis of Induced Circulatory Failure. Am. J. Physiol., 169, (1952): 434–441.

[3] Burwell, C. S., and Flickinger, D.: Obstructing Pericarditis. Arch. Int. Med., 56, (1935): 250–257.

[4] Burwell, C. S., and Strayhorn, W. D.: Concretio Cordis. Arch. Surg., 24, (1932): 106–119.

[5] Doyle, J. T., Patterson, J. L., Warren, J. V., Detweiler, D. K., and Reynolds, M.: Hemodynamic Observations on the Cow. Fed. Proc., 17, Part I, (1958): 38.

[6] Fisher, E. W., and Dalton, R. G.: Cardiac Output in Cattle. Nature, London, 183, (1959): 829.

[7] Fowler, W. S., Blackburn, C. M., and Helmholz, H. F.: Determination of Basal Rate of Oxygen Consumption by Open and Closed Circuit Methods. J. Clin. Endocrinol., 17, (1957): 786–796.

[8] Greisheimer, E. M., Ellis, D. W., Baier, H. N., Ring, G. C., Makarenko, L., and Graziano, J.: Cardiac Output by Cuvette Oximeter under Thiopental. Am. J. Physiol., 175, (1953): 171–172.

[9] Hemingway, A.: Variability of Cardiac Output and Respiration of Dogs During Pentobarbital Anesthesia. Fed. Proc., 19, Part I, (1960): 117.

[10] Isaacs, J. P., Carter, B. N., and Haller, Jr., J. A.: Experimental Pericarditis: The Pathologic Physiology of Constrictive Pericarditis. Bull. Johns Hopkins Hosp., 90, (1952): 259–300.

[11] Lusk, G.: The Elements of the Science of Nutrition. 4th ed. W. B. Saunders Co., Philadelphia, 1928.

[12] Lyons, R. H., and Burwell, C. S.: Induced Changes in the Circulation in Constrictive Pericarditis. Brit. Heart J., 8, (1946): 33–46.

[13] Post, R. S.: Decrease of Cardiac Output by Acute Pericardial Effusion and Its Effect on Renal Hemodynamics and Electrolyte Excretion. Am. J. Physiol., 165, (1951): 278–284.

[14] Schambye, P.: Bestemmelse af hjertets minutvolumen hos far med Hamilton's injektionsmetode (English summary). Nord. Vet.-med., 4, (1952): 1148–1172.

[15] Stowe, C. M., and Good, A. L.: Estimation of Cardiac Output in Calves and Sheep by the Dye and Fick Oxygen Techniques. Am. J. Physiol., 198, (1960): 987–990.

[16] Wilson, R. H., Hoseth, W., Sadoff, C., and Dempsey, M. E.: Pathologic Physiology and Diagnostic Significance of the Pressure Pulse Tracings in the Heart in Patients with Constrictive Pericarditis and Pericardial Effusion. Am. Heart J., 48, (1954): 671–683.

[17] Zuntz, N., and Hagemann, O.: Untersuchungen über den Stoffwechsel des Pferdes bei Ruhe und Arbeit. Landw. Jahrb., 27, Supplement, 3, (1898): 371.

SUMMARIO IN INTERLINGUA

Estimation del Rendimento Cardiac in Animales Domestic per Medio del Technica Directe de Fick, con Observationes in Re un Caso de Pericarditis Traumatic

Le rendimento cardiac de plure anesthesiate animales representative (presumitemente) de quatro species de bestial major esseva determinate per medio del technica directe a oxygeno de Fick. A generalmente parlar, le rendimento cardiac de iste animales esseva inter le limites de 70 e 150 ml/kg/min, durante que le indices cardiac variava inter approximativemente 2 e 9. Le majoritate del valores esseva in le vicinitate de 4 a 6. In un juvenca, afficite de pericarditis traumatic, un relativemente basse rendimento cardiac e indice cardiac esseva trovate, insimul con elevation del tension jugular e dextero-ventricular.

Uterine Healing After Cesarotomy in Goats

R. P. S. Tyagi, B.V.Sc., A.H., M.S., and W. V. Lumb, D.V.M., Ph.D.

SUMMARY

In cesarotomies on 8 goats, 18 uterine incisions were made. Biopsies were performed at intervals postoperatively, and macroscopic and microscopic observations were made. In 1 goat, two uterine incisions were sutured with No. 50 nylon, using a single row of simple, interrupted Lembert stitches on one, and a single row of continuous lock stitches on the other. Single rows of stitches were used only on this goat and did not produce a safe closure. The uterine incisions on the remaining goats were sutured with either catgut or nylon, using either a double row of continuous lock stitches, or two rows of simple, interrupted Lembert stitches.

On microscopic examination, it was observed that the edges of the wound throughout the entire line of incision were held together by early proliferation of fibroblasts and blood capillaries. Definite scar tissue could be seen 14 to 27 days postoperatively. Muscle regeneration could not be observed in the healing process.

When catgut suture was used, there was severe tissue reaction in 4 goats and omental adhesions in 2. No such reactions occurred with the use of nylon.

INTRODUCTION

In the past decade, due to improvements in anesthetic and surgical techniques, cesarotomy has become an established and important procedure in veterinary obstetric surgery. Since the hazards of this operation have significantly decreased, indications for its use have broadened to include many complications of pregnancy and labor which were formerly managed by vaginal delivery. However, it must be emphasized that cesarotomy is not only associated with some immediate risk, but it also predisposes the patient to certain dangers if future pregnancies develop. The condition of the healed uterus after cesarotomy is an important factor in the safety of future pregnancy. The purpose of this article is to report observations on the healing process of uterine incisions made during cesarotomy in goats.

Review of Literature

Healing of incisions in the human uterus has been thoroughly studied by many investigators.[2, 6, 14, 16] In earlier literature, authors are divided into two schools of thought. Baunfunwold *et al.*, cited by Audebert and Williams,[2, 16] reported complete muscle regeneration in the scar after cesarotomy, whereas Couvelaire[6] doubted that the reparative processes could occur without fibrous tissue formation. Fruhinshols, Lauvray, and Nicoletti, cited by Hindman,[9] agreed with the latter principle. Schwarz and Paddock[15] appear to have settled the issue in their histologic study of the scar. They demonstrated the laying down of fibrin and fibroblasts along the line of incision in the early healing process. The results of a series of experiments by these investigators on the uteri of pregnant guinea pigs at term confirmed that the process of healing was by scar tissue formation. Their observations have not been contradicted.

The healing process of the uterine wall in domestic animals after cesarotomy has received little attention until recently, judging by the relatively meager information appearing in the literature. In 1957, Cheli[4] studied the healing process in bovine, ovine and canine uteri after cesarotomy at different stages of pregnancy. He also observed the formation of cicatricial tissue at the site of the scar.

The process of healing was considerably affected by the different suture techniques and materials reported by the various authors.[2, 6, 7, 11–14]

Materials and Methods

Pregnant adult goats were used in this study. Weight varied from 85 to 115 lb. each. Near term, they were kept in separate stalls and given a ration of hay and a commercial ground dairy feed. Surgery was performed when the usual physical signs of approaching parturition appeared. The goats

Received for publication Aug. 31, 1960.

From the Department of Surgery and Medicine, Michigan State University, East Lansing.

This paper is an extract from the thesis submitted by the senior author in partial fulfillment of the requirements for the M.S. degree, at the College of Veterinary Medicine, Michigan State University, East Lansing.

were fasted overnight, and an antiferment* was given via stomach tube one hour before surgery. Ten minutes before the operation, a tranquilizer, trifluprom azine hydrochloride,** was injected intravenously, at the rate of 1 mg. per pound of body weight, as a preanesthetic.

An ultra-short-acting barbiturate, thiamylal sodium,† was used as a general anesthetic in goat 5. In the rest of the goats, procaine hydrochloride was given, using a paravertebral-lumbar nerve-block technique. The animal was placed on the operating table in right lateral recumbency, and the left paralumbar fossa was clipped and scrubbed with liquid germicidal detergent. The operative site was masked with sterile drapes, and a vertical 6-inch flank incision was made through the skin, muscles, and peritoneum midway between the last rib and the tuber coxa.

The uterus was located and brought to the outside through the incision. A 5-inch incision was made into each horn near the body of the uterus in all the animals except goat 10, in which the uterus was incised at four sites. The fetuses were delivered immediately, dried with towels, and placed in a cardboard box fitted with light bulbs to provide warmth. After extraction of the kids, the protruding fetal membranes were returned to the uterine horns. In goat 5, the uterine incisions were closed with size 00 chromic catgut using a double row of continuous lock stitches. In goat 10, 13, and 18, the uterine incision in one horn was sutured with size 00 chromic catgut using a double row of continuous lock stitches, and in the other, using a double row of interrupted Lembert stitches. In goats 1, 2, and 21, the uterine incisions were closed with No. 50 nylon, using a double row of continuous lock stitches in one horn and a double row of interrupted Lembert stitches in the

* Turcapsol, produced by Pitman-Moore Co., Indianapolis, Ind.

** Vetame, produced by E. R. Squibb and Sons, New Brunswick, N.J.

† Surital, produced by Parke, Davis and Co., Detroit, Mich.

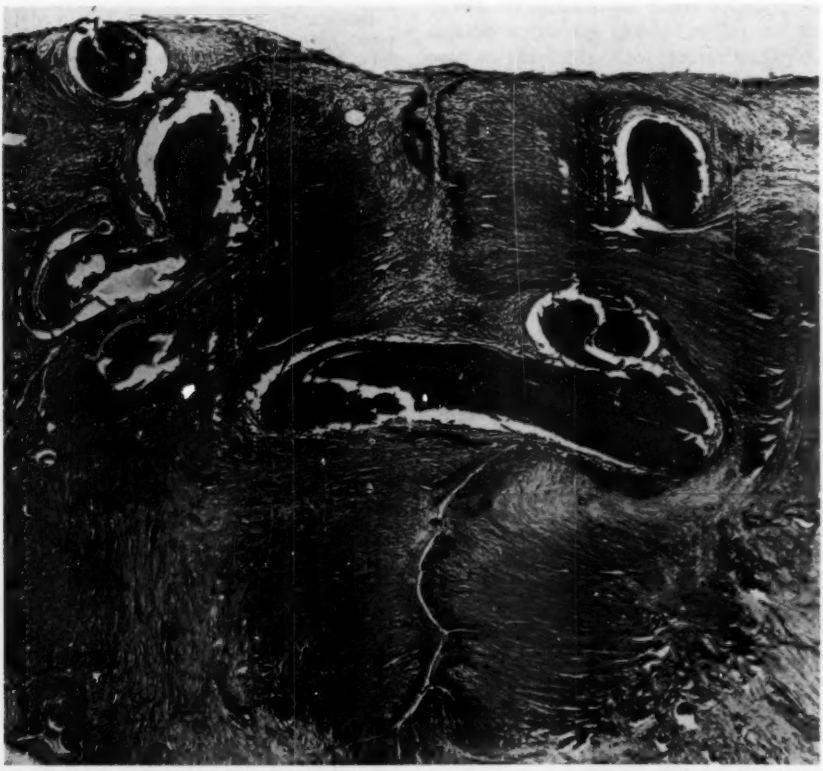


Fig. 1—Photomicrograph of uterine tissue from goat 10 on postoperative day 14. Two rows of continuous lock stitches of 00 chromic catgut were used in uterine closure. The incision can be seen running vertically through the center. Large dark masses are catgut surrounded by phagocytes; x 10.

other. In goat 3 only, a single row of continuous lock stitches was used in one horn and a single row of interrupted Lembert stitches in the other. In all the goats, the incisions in the peritoneum and muscles of the abdominal wall were closed with simple, interrupted, nonabsorbable sutures,* and the skin was brought in apposition with similar sutures.

The healing process of the uterine wounds was observed 3, 14, 18, 19, 21, 26, and 27 days after uterine closure. At the end of the designated period, the uterus was exposed under aseptic conditions, and a biopsy was performed. One square inch of uterine tissue was obtained from the site of the scar and placed in 10% formalin. Sections were made for microscopic examination.

Results

In this study, a general anesthetic was used in 1 goat. It did not adversely affect the mother, but it had a depressive effect on the kids. To avoid this difficulty in subsequent cases, a combination of triflupromazine hydrochloride as a preanesthetic and procaine hydrochloride as a local anesthetic was used. This combination produced satisfactory anesthesia and caused no ill effects in the kids.

The lower left flank approach was most satisfactory. In the midline approach, the udder was a considerable hindrance. Involution of the uterus after the removal of the fetuses was not so rapid as reported by other investigators,[1, 4] and thus no difficulty was encountered in suturing the incision of the uterine horns. However, difficulty was encountered in separating the fetal membranes from the uterine mucosa. To allow them to separate naturally, the membranes were left inside the uterus after removing

* Vetafil, produced by Bengen & Co., Hannover, Western Germany.

Fig. 2—Higher magnification of incision shown in Figure 1. Fibroblastic proliferation is evident between the edges of the incision; x 50.

the kids. Fetal membranes were usually expelled postoperatively within 12 hours.

The character of healing was studied in 18 uterine incisions. Macroscopically, biopsy revealed that healing was complete in all animals except goat 3 which died of uterine infection after surgery. Most animals had a clear scar with no adhesions to adjacent organs, but there were adhesions in goats 5 and 10. In goat 10, biopsy was made at two intervals, 14 and 19 days postoperatively. At the first biopsy, the wound was free of adhesions, but at the second, extensive adhesions were observed between the wall of the uterus and the omentum.

Microscopically, the line of incision could be followed easily in the tissue obtained from goats 10 and 13 (Fig. 1, 2) at 14 and 18 days after surgery, respectively. In the other goats, this line could not be observed because complete healing had occurred. However, a preponderance of fibrous tissue denoted the probable site of the incision. Considerable differences were observed in the tissue reactions to different suture materials (Fig. 3, 4). Around catgut sutures, a marked reaction was characterized by the presence of blood and numerous leukocytes. Around nylon sutures, there was little or no tissue reaction (Fig. 5, 6). No evidence of smooth muscle regeneration was observed.

Discussion

The histologic characteristics of the healing wound largely reflect the strength of

Fig. 3—Uterine tissue from goat 1 on postoperative day 14. Two rows of simple, interrupted stitches of No. 50 nylon were used in uterine closure. The incision runs vertically through the center of the photomicrograph and is almost indiscernible. There is little peritoneal reaction. The straight line through the tissue is an artifact; x 55.

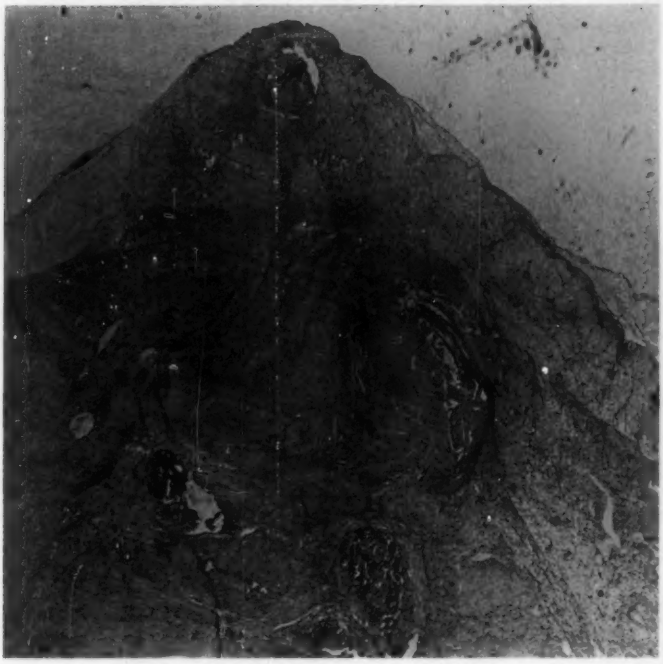


Fig. 4—Uterine tissue from goat 21 on postoperative day 21. Two rows of continuous lock stitches of 00 chromic catgut were used in uterine closure. An intense peritoneal proliferation surrounds the catgut near the surface of the uterus. The line of incision cannot be definitely distinguished; x 40.

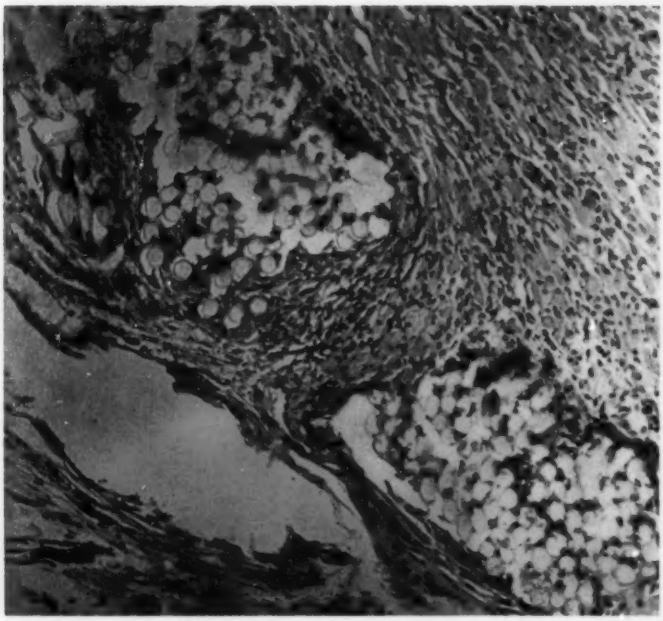


Fig. 5—Nylon suture (No. 50) in goat uterus 19 days postoperatively. Notice the honeycomb appearance of the nylon and the lack of surrounding tissue reaction; x 40.

TABLE 1—Effects of Various Suture Materials and Techniques on the Healing of the Uterine Wall in Goats After Cesarotomy

Goat No.	Suture material	Suture technique A. Right horn B. Left horn	Biopsy (No. days postoperation)	Gross appearance	Microscopic appearance
3	Nylon	A. Single row of simple, interrupted Lembert stitches. B. Single row of continuous lock stitches.	3	A. A hole 1 cm. in diameter in the uterine incision. No adhesions. B. No adhesions. Stitches intact.	No healing. Infection present. Line of cleavage clearly defined. No healing.
1	Nylon	A. Two rows of simple, interrupted Lembert stitches. B. Two rows of continuous lock stitches.	14	A. No adhesions. B. No adhesions.	Complete healing. No infection. Little reaction in the tissue. Line of incision could not be distinguished.
10	Catgut	A. Two rows of simple, interrupted Lembert stitches. B. Two rows of continuous lock stitches.	14	A. No adhesions. B. No adhesions.	Healing in progress. No infection. Marked reaction around sutures. Line of incision could be followed in the tissue.
13	Catgut	A. Two rows of simple, interrupted Lembert stitches. B. Two rows of continuous lock stitches.	18	A. No adhesions. B. No adhesions.	Healing complete. Extensive reaction around sutures. Line of incision could be distinguished in tissue.
10	Catgut	A. Two rows of simple, interrupted Lembert stitches. B. Two rows of continuous lock stitches.	19	A. Extensive adhesions around sutures. B. Extensive adhesions around sutures.	Healing complete. No infection. Marked reaction around the sutures. Line of incision not clearly defined.
2	Nylon	A. Two rows of simple, interrupted Lembert stitches. B. Two rows of continuous lock stitches.	19	A. No adhesions. B. No adhesions.	Healing was good. Little reaction in the tissue. No reaction around the sutures. Line of incision could not be distinguished.
21	Nylon	A. Two rows of simple, interrupted Lembert stitches. B. Two rows of continuous lock stitches.	21	A. No adhesions. B. No adhesions.	Some healing at a few places along the line of incision. No tissue reaction. The incision line could not be definitely distinguished.
5	Catgut	A. Two rows of continuous lock stitches. B. Two rows of continuous lock stitches.	26	A. Slight adhesions. B. Slight adhesions.	Infection present. Extensive tissue reaction. Complete healing. Incision line could not be distinguished.
18	Catgut	A. Two rows of simple, interrupted Lembert stitches. B. Two rows of continuous lock stitches.	27	A. No adhesions. B. No adhesions.	Healing complete. Phagocytic action in tissue. Incision line could not be distinguished.

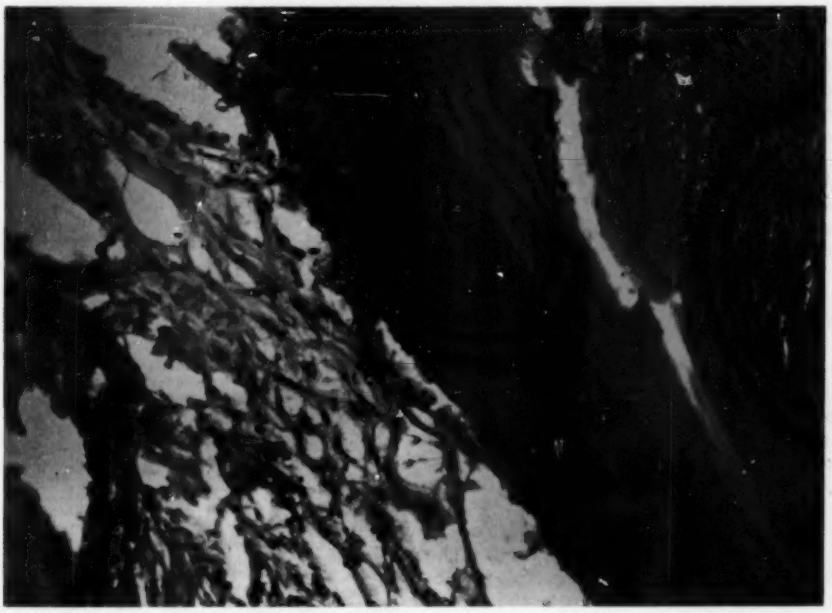


Fig. 6—Chromic 00 catgut suture in goat uterus 27 days postoperatively. Phagocytes are infiltrating the catgut along its surface; x 50.

the scar. Many differences of opinion on this subject have been expressed in the literature. A large group of obstetricians believes that healing of the uterine incisions occurs through the formation of scar tissue, while another group firmly believes that healing takes place entirely through the process of muscle regeneration. The ability of smooth muscle fibers to grow and regenerate has been reported by many authors.[1, 7, 16] However, no smooth muscle regeneration could be observed in this study. On the other hand, in all eight uteri of this series, microscopic study revealed fibroblasts along the line of incision, with the scar composed mainly of connective tissue. These findings agreed with those of Schwarz and Paddock [15] who observed fibroblasts along the incision line and showed that healing was the result of proliferation of fibrous tissue. Cheli [4] also observed the formation of cicatricial tissue in uteri of cattle and sheep after cesarotomy.

This study of 18 uterine scars showed results similar to those reported by Couvelaire and Losee,[5, 10] in that the deposition of a considerable band of fibrin between the cut edges formed the basis from which healing took place. Fibroblastic proliferation, evident in all goats, could be considered normal healing, indicating that the early process was similar to that in the guinea pig and rabbit, described by Schwarz and Paddock.[15]

In this study, three methods were used to close the uterine incisions. In goat 3, a single layer of interrupted Lembert stitches of No. 50 nylon was used to close the wound in one horn. In this case, the sutures gave way, leaving a gap of about 1 cm., and the animal died three days after surgery. Postmortem examination revealed that uterine contents had spilled into the abdominal cavity and peritonitis had developed. From this observation, it would appear that a single layer of No. 50 nylon did not produce a safe closure. In the remaining animals, a double row of continuous lock stitches or a double row of interrupted Lembert stitches was used to close the uterine incisions. Both types of stitches gave good results, and healing was satisfactory in all cases. Two essential points for producing a better scar were observed. One was the inclusion of the entire wall of the uterus in the suture in order to secure

proper coaptation of the entire length of incision. The other was the prevention of infection. There is some difference of opinion as to whether the uterine wound should be closed with interrupted or continuous stitches. Greenhill and Bloom [8] observed that closure with interrupted stitches yielded better scars than closure with continuous stitches. Others [12,13] have preferred continuous stitches. In this study, both types produced good results, but since the continuous lock-stitch technique was faster, it was preferred.

Suture materials are an important factor in a closure. The fact that a wide variety of suture materials has been used by many investigators in closing the uterine incision is sufficient indication that none is entirely satisfactory. Plain and chromic catgut, silkworm-gut, kangaroo tendon, silk, nylon, and metallic wire have all been employed.

In this series of operations, No. 50 nylon and 00 chromic catgut were used. The former yielded more satisfactory results than the latter. With catgut, there was marked reaction around the sutures in most cases, and in 2 goats, 5 and 10, omental adhesions developed along the incision line, probably as a result of stiff knots irritating the surrounding tissue. With nylon, little or no reaction occurred around the suture material and the uterine scar at the time of biopsy was free of adhesions. This was perhaps due to the fact that, since nylon has great tensile strength, fine materials could be used, thereby reducing the size of the foreign body. Further advantages of nylon are that it does not possess the power of capillary attraction, and it has a smooth, nonirritating surface which causes less tissue reaction.

According to Gamble,[7] adhesions after cesarotomy are common. Similar inferences were not justifiable in this study since adhesions were found in only 2 goats.

References

[1] Arthur, G. H.: Cesarean Section in the Ewe. Vet. Rec., 64, (1952): 482–486.

[2] Audebert, M.: Cesarean Section Scar. Gynéc. et Obst., 7, (1923): 487.

[3] Babcock, W. W.: An Improved Method of Uterine Closure in High Classical Cesarean Section. Am. J. Obst. & Gynec., 43, (1942): 303.

[4] Cheli, R.: The Healing Process in the Animal Uterus After Cesarean Section. Nuova vet., 33, (1957): 381–427.

[5] Couvelaire, A.: Rupture de la cicatrice d'une ancienne opération Césarienne, survenue à la fin d'une grossesse compliquée d'hydramnios. Ann. Gynéc. et Obst., 2. s., (1906): 148–164.

[6] Davis, E. P.: The Uterus After Cesarean Section. Am. J. Obst. & Gynec., 4, (1922): 335–350.

[7] Gamble, T. O.: A Clinical and Anatomical Study of Fifty-One Cases of Repeated Cesarean Section. Bull. Johns Hopkins Hosp., 33, (1922): 93–105.

[8] Greenhill, J. P., and Bloom, E.: Histological Study of Uterine Scars After Cervical Cesarean Section. J. Am. M.A., 92, (1929): 21–26.

[9] Hindman, D. H.: Pelvic Delivery Following Cesarean Section. Am. J. Obst. & Gynec., 2, (1948): 273–285.

[10] Losee, J. P.: The Cesarean Scar, an Anatomical Study. Bull. Lying-In Hosp., New York, 2, (1918): 228–240.

[11] Mackinnon, M. M., and Bayliss, J.: Cesarean Section in the Ewe. Vet. Rec., 64, (1952): 512–513.

[12] Milne, F. J.: Cesarean Section in the Ewe. Vet. Rec., 63, (1951): 617.

[13] Potter, G. M., and Norman, W. E.: An Improved Method of Uterine Closure in High Classical Cesarean Section. Am. J. Obst. & Gynec., 43, (1942): 303–306.

[14] Schmitz, H. E., and Gajewski, G. J.: Vaginal Delivery Following Cesarean Section. Am. J. Obst. & Gynec., 61, (1951): 1231–1241.

[15] Schwarz, O. H., and Paddock, R.: The Cesarean Scar. Am. J. Obst. & Gynec., 10, (1925): 153–171.

[16] Williams, J. W.: A Histological Study of Fifty Uteri Removed at Cesarean Section. Bull. Johns Hopkins Hosp., 28, (1917): 335–343.

[17] Williams, J. W.: Regeneration of the Uterine Mucosa After Delivery with Special Reference to the Placental Site. Am. J. Obst. & Gynec., 22, (1931): 664–696.

SUMMARIO IN INTERLINGUA

Resanation Uterin post Cesarotomia in Capras

In cesarotomias in 8 capras, 18 incisiones uterin esseva facite. Biopsias esseva executate a intervallos post le operation, e observationes macro- e microscopic esseva facite. In 1 capra, duo incisiones uterin esseva saturate con nylon numero 50, le prime con un sol linea de simple, interrumpite punctatas Lembert, le altere con un sol linea de continue punctatas nodate. Le uso de un sol linea de simple, interrumpite punctatas non esseva repetite in ulle del altere capras. Illo non resultava in un clauditura secur. Le incisiones in le altere capras esseva suturate con catgut o con nylon, con le uso de un duple linea de continue puncturas nodate o de duo lineas de simple, interrumpite punctatas Lembert.

In le examine microscopic il esseva observate que le margines del vulnere al longo del integre incision esseva reunite per un precoce proliferation de fibroblastos e capillares sanguinee. Evidentia definite de tissu cicatrisante poteva esser vidite inter 14 e 27 dies post le operation. Regeneration muscular non poteva esser observate in le processo resanatori.

Quando catgut esseva usate in le suturas, il occurreva sever reactiones tissular in 4 capras e adhesiones omental in 2. Nulle tal reactiones esseva observate post le uso de nylon.

Critical Tests with the Organic Phosphate Insecticide, Dimethoate, Against *Gastrophilus spp.* in the Horse, with Observations on Its Anthelmintic Action

J. H. Drudge, D.V.M., Sc.D.; S. E. Leland, Jr., Ph.D.; Z. N. Wyant, B.S., A.M.; G. W. Elam; and E. T. Lyons, B.S., M.S.

SUMMARY

Bot removal and anthelmintic activity of the organic phosphate, dimethoate, was evaluated in a series of 21 critical tests on horses.

Selective action against *Gastrophilus nasalis* was quite evident. In tests with single doses via stomach tube, complete removal of both second and third instar *G. nasalis* resulted from doses as low as 20 mg./kg. In contrast, only limited action against third instar *Gastrophilus intestinalis* resulted from single doses as high as 80 mg./kg., which were toxic for horses. Second instars of *G. intestinalis* were more susceptible; in the aggregate, 80% were removed by single doses of 20 to 80 mg./kg.

Administration in the feed markedly increased the efficacy against bots, and three daily doses at the rate of 20 mg./kg. removed more than 90% of both instars of both species. However, individual variation in the acceptance of medicated feed made this method of administration generally difficult and unpredictable, and mild toxic reaction accompanied the treatment.

The anthelmintic activity of dimethoate was limited but was generally enhanced by administration in the feed. *Parascaris equorum* was refractory to single doses, but an average of 70% were removed by three 20 mg./kg. doses in the feed. Limited data also indicated that adult *Oxyuris equi* were more effectively removed by multiple daily doses in the feed. Post-treatment changes in strongyle egg counts were quite variable and, as an index of anti-strongyle activity, showed no consistent and striking reductions even in the feeding trials.

INTRODUCTION

Numerous investigations[1] have been made in recent years on the insecticidal activity of the organic phosphates or the so-called systemic insecticides. In the domestic animals, special attention has been directed toward the control of *Hypoderma spp.* in cattle, and *Oestrus ovis* in sheep. Activity against horse bots, *Gastrophilus spp.*, has also been investigated,[2,4-6] but to a lesser extent. More recently, the anthelmintic action of these compounds in cattle and sheep has been under extensive study.[1]

Dimethoate* is active against *Hypoderma spp.*[8] and *Dermatobia hominis*[7,9] in cattle and highly effective against *Oestrus ovis* in sheep.[11] Our preliminary trials[4,5] have indicated it is also quite active against *Gastrophilus spp.* of the horse. In contrast, other investigators[6] reported, "dimethoate failed to control bots in initial tests" in their studies on eight organic phosphates.

The present report covers all of the critical tests that have been completed for the evaluation of dimethoate against bots, and includes observations on the anthelmintic action of dimethoate in the horse.

Materials and Methods

The 21 test animals used in this investigation were of mixed light horse breeding, and all of the parasitic infections were naturally acquired. Two species of bots, *Gastrophilus intestinalis* and *Gas-*

Received for publication March 13, 1961.

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington. Published by permission of the director of the station.

The study was supported in part by grants from the Agricultural Division of American Cyanamid Company, and the Grayson Foundation, Inc.

* American Cyanamid compound CL 12,880: 0,0-dimethyl-S(N-methylcarbamoyl-methyl) phosphorodithioate.

trophilus nasalis, were present. Removal activity against these was of special interest; however, the fecal examinations also detected the passage of *Parascaris equorum* and adult female pinworms, *Oxyuris equi*. The necropsy was routinely made after a four- or five-day post-treatment fecal collection interval and included a search of the stomach for bots, the small intestine for ascarids, and the large intestine for pinworms that remained after treatment. Activity against strongyles was evaluated by pre- and post-treatment egg counts on feces, using the same techniques outlined earlier for similar tests in horses.[8]

Single doses of the drugs, given via stomach tube, were tested in 11 horses; dosage rates of 10, 20, 40, 60, and 80 mg./kg. of body weight were used. In these animals, the grain ration was withheld on the morning of treatment; there were no other fasting procedures. Two drug formulations, a 50% parenteral solution and a 25% wettable powder, were used.

Subcutaneous administration of the 50% parenteral solution was tested in 2 yearlings at 20 mg./kg. and at 30 mg./kg. in 1 mare.

Multiple daily doses were studied in 7 horses. Two of these used were largely palatibility trials; however, some data were obtained on possible action against strongyles. Three preparations of the drug, a 31% drench powder, a 25% wettable powder, and a 10% premix were used. For administration, each dose of the powder was mixed with approximately one-half gallon of dampened oats. Varying periods of time, from one to 24 hours, were required for the medicated grain to be completely consumed. The 31% drench powder was not as unpalatable as the 25% wettable powder or the 10% premix. All of the horses were given a preliminary exposure to the drug's odor by placing a minute amount in the feed box for a day or two before actual treatment was started.

Observations for clinical signs of toxicosis were made on all animals. Pre-and post-treatment plasma cholinesterase determinations were made on 1 horse by means of Michel's method.[10]

Results

The removal of bots in 11 horses treated with single doses of dimethoate via stomach tube is summarized (Table 1). At 10 mg./kg., no action against either species of bots was recorded. Dosages of 20 mg./kg. and above were, with one exception, 100% effective in the removal of both second and third instar *G. nasalis*. In contrast, the action of the same dosage rates on *G. intestinalis* was incomplete and quite variable. Second instars of this species were more susceptible than the third instars and, in the aggregate, the average removal percentages were 80 and 11, respectively, for the range of doses from 20 to 80 mg./kg. Complete removal was observed in only 1 horse, and this was at 60 mg./kg.

At single oral dosages of 20, 40, and 60 mg./kg., a 25% wettable powder formulation was consistently more effective in removing both second and third instar *G. intestinalis* than the 50% parenteral solution. Both formulations were equally effective against *G. nasalis*.

Data pertaining to bot removal by multiple daily doses administered in the grain ration are outlined (Table 2). Third instars of *G. intestinalis* were the most difficult to remove, and a level of action above 90% required doses of 20 mg./kg. for three

TABLE 1—Critical Test Data on the Efficacy of Single Doses of Dimethoate Against Second and Third Instar Gastrophilus intestinalis and Gastrophilus nasalis

Horse		Treatment		*G. intestinalis*						*G. nasalis*					
				No. removed		No. left		% efficacy		No. removed		No. left		% efficacy	
Number	Weight (kg.)	Rate (mg./kg.)	Preparation	2nd	3rd	2nd	3rd	2nd	3rd	2nd	3rd	2nd	3rd	2nd	3rd
SINGLE DOSE VIA STOMACH TUBE															
16	343	10	50% Sol.	0	0	0	80	....	0	0	0	0	58	....	0
661	245	20	50% Sol.	0	0	1	254	0	0	0	0	0	0	....	....
742	496	20	25% WP	23	24	48	93	32	21	2	14	2	0	50	100
740	284	20	25% WP	17	1	2	34	89	3	5	0	0	0	100	....
627	230	40	50% Sol.	0	6	0	265	....	2	0	12	0	0	....	100
706	334	40	25% WP	78	6	13	11	86	35	4	26	0	0	100	100
738	475	40	25% WP	1	1	1	1	50	50	1	3	0	0	100	100
693	339	40	25% WP	61	12	26	78	70	13	9	58	0	0	100	100
5	260	60	50% Sol.	0	40	0	40	....	50	0	144	0	0	....	100
702	311	60	25% WP	190	20	0	0	100	100	25	53	0	0	100	100
620	200	80	50% Sol.	0	0	0	117	....	0	0	32	0	0	....	100
SINGLE DOSE, SUBCUTANEOUSLY															
630	245	20	50% Sol.	1	0	0	115	100	0	0	37	0	22	....	76
705	320	20	47% Sol.	0	0	0	80	....	0	0	1	0	1	....	50
749	505	30	47% Sol.	0	2	0	107	....	2	0	10	0	0	....	100

Sol. = solution, parenteral; WP = wettable powder.

TABLE 2—Critical Test Data on the Efficacy of Multiple Daily Doses of Dimethoate in Feed Against Second and Third Instar Gastrophilus intestinalis and Gastrophilus nasalis

Horse		Treatment		*G. intestinalis*						*G. nasalis*					
				No. removed		No. left		% efficacy		No. removed		No. left		% efficacy	
Number	Weight (kg.)	Rate (mg./kg.)	Preparation	2nd	3rd	2nd	3rd	2nd	3rd	2nd	3rd	2nd	3rd	2nd	3rd
622	332	3 x 10	31% DP	15	0	0	311	100	0	0	0	0	48	.....	0
15	384	5 x 10	10% PM	0	95	0	62	.....	61	0	157	0	0	.....	100
698	309	2 x 20	25% WP	58	16	0	36	100	31	0	10	0	0	.....	100
S 16	473	3 x 20	31% DP	x	x	0	2	.....	....	x	x	0	0	.....	.....
M 17	536	3 x 20	31% DP	x	x	x	x	.....	....	x	x	x	x	.....	.....
625	315	3 x 20	31% DP	44	75	0	7	100	91	6	42	0	0	100	100
629	334	4 x 20	31% DP	44	82	0	2	100	98	42	176	0	0	100	100

PM = Two doses of 10% premix plus four doses of 31% drench powder; DP = drench powder; WP = wettable powder; x = no observation made.

consecutive days. In comparison, second instar *G. intestinalis* and third instar *G. nasalis* were more susceptible. Regimens of 10 mg./kg. for three and six days, respectively, were sufficient for 100% removal. Evaluation of the smaller doses against second instar *G. nasalis* was not obtained because of their absence in these animals.

Observations on the anthelmintic action showed that single doses were devoid of action on ascarids and only partially effective against mature *O. equi* (Table 3). In contrast, effective removal of both of these species was recorded in the multiple dose trials in which three or four daily 20 mg./kg. doses were fed (Table 4). Activity against strongyles was based on changes in fecal egg counts after treatment, and consistently high level reductions were not observed. In two of the three tests with single doses at the toxic levels of 60 and 80 mg./kg., reduction of strongyle eggs per gram (EPG) was 75% or above (Table 3). Likewise, in two of the three multiple dose tests at 20 mg./kg., reductions of 60% or above were recorded (Table 4). The consistency of the feces was softened by these treatments and tended to be a dilution factor in the post-treatment egg counts.

Toxic manifestations of the drug were observed in several of the horses given single doses at the higher levels. The untoward effects were moderate at 60 mg./kg. and consisted of softening of the feces for a day or two after treatment. At 80 mg./kg., a severe reaction was produced; the signs were severe abdominal pain, frequent watery defecations, rapid weakening, depression, and loss of appetite during the

TABLE 3—Critical Test Data on Efficacy of Single Doses of Dimethoate Against Ascarids and Pinworms and Fecal Egg Count Data on Anti-Strongyle Actviity

Horse No.	Dosage (mg./kg.)	*Parascaris equorum*						*Oxyuris equi* ♀			Strongyle eggs/Gm.		
		No. removed		No. left		% efficacy		No. removed	No. left	% efficacy	No. before	No. after	% change
		Immat.	Mat.	Immat.	Mat.	Immat.	Mat.						
SINGLE DOSE VIA STOMACH TUBE													
16	10	0	0	0	0	..	..	0	12	0	640	700	+ 9
661	20	0	0	0	3	..	0	4	14	22	380	400	+ 5
742	20	0	0	0	0	..	..	0	0	....	990	180	—82
740	20	0	0	x	x	..	..	0	x	....	x	x	.....
627	40	0	0	0	22	..	0	6	13	32	80	90	+13
706	40	0	0	0	0	..	..	0	x	....	650	840	+29
738	40	0	0	0	0	..	..	0	x	....	x	x	.....
693	40	0	0	0	0	..	..	1	x	....	1,450	2,290	+58
5	60	0	0	0	4	..	0	0	0	....	3,090	760	—75
702	60	0	0	0	0	..	..	2	x	....	580	120	—79
620	80	0	0	0	22	..	0	0	0	....	400	380	— 5
SINGLE DOSE, SUBCUTANEOUSLY													
630	20	0	0	0	6	..	0	27	3	90	1,740	1,070	—39
705	20	0	0	0	4	..	0	0	0	....	130	60	—54
749	30	0	0	0	0	..	..	0	0	....	40	50	+20

x = no observation made.

first day after treatment. The horse remained quite weak and depressed for an additional day, during which little hay or grain was consumed and fecal output was much reduced. By the third day, the appetite had returned and the feces became nearly normal in consistency and amount. In comparison with the pretreatment value, the plasma cholinesterase activity was markedly reduced (67%) within four hours after treatment and remained at this low level during the four-day post-treatment observation period.

In the multiple daily dose feeding trials, the only apparent untoward effect was the changing of the feces to a soft, mushy consistency. This was observed only in the trials at 20 mg./kg. It generally started on the second day of treatment and persisted for three or four days after the last dose was fed. About half of the animals were reluctant to eat the medicated grain, and difficulty was encountered in getting them to consume the prescribed course of treatment. This was observed at both dose rates and at both the beginning and ending of the various treatment periods.

TABLE 4—Critical Test Data on the Efficacy of Multiple Daily Doses of Dimethoate Against Ascarids and Pinworms and Fecal Egg Count Data on Anti-Strongyle Activity

Horse No.	Dosage (mg./kg.)	*Parascaris equorum*						*Oxyuris equi* ♀			Strongyle eggs/Gm.		
		No. removed		No. left		% efficacy		No. removed	No. left	% efficacy	No. before	No. after	% change
		Immat.	Mat.	Immat.	Mat.	Immat.	Mat.						
622	3 x 10	0	0	0	0	..	..	0	3	0	1,570	1,800	+15
15	6 x 10	0	0	0	1	..	0	0	x	..	2,020	880	−56
698	2 x 20	0	0	0	0	..	..	1	x	..	x	x	.....
S 16	3 x 20	x	x	x	x	..	..	x	x	..	1,065	430	−60
M 17	3 x 20	x	x	x	x	..	..	x	x	..	385	450	+17
625	3 x 20	1	6	0	3	100	67	0	0	..	x	x	.....
629	4 x 20	14	7	0	1	100	88	5	0	100	1,330	410	−69

x = no observation made.

Discussion

The bot removal action of dimethoate showed definite species selectivity in these tests. *Gastrophilus nasalis* was relatively susceptible and was effectively removed by single doses as low as 20 mg./kg. *Gastrophilus intestinalis* was more refractory and was only partially removed by single doses, including those in the toxic range (60 to 80 mg./kg.). A selective activity against *G. nasalis* was previously reported [3] for the piperazine-carbon disulfide complex, although subsequent unpublished observations have not borne out the earlier findings. Another of the organic phosphates, Bayer L 13/59,* has been reported [6] as only slightly more effective against *G. nasalis* than *G. intestinalis*. The location of *G. nasalis* just beyond the pylorus in the small intestine would favor contact with the drug; this physical factor may be partially accountable for the selective action. However, this tends to be refuted by the tests on toluene,[12] where *G. intestinalis* was more effectively removed than *G. nasalis*. Thus, the present observations on dimethoate are the first to show a definite species-selective action on bots in which *G. nasalis* is more susceptible than *G. intestinalis*.

There was also selective action in the more effective removal of second instars of *G. intestinalis* than the third instars of this species. However, even single oral doses did not result in their complete removal except in one animal given 60 mg./kg., which was in the toxic range. Greater susceptibility of the earlier instars of both *Hypoderma spp.* and *Oestrus ovis* has also been reported.[8,11] A similar differential action of dimethoate on *G. nasalis* was not observed in this study because of the effective removal of both stages of this species at 20 mg./kg., which was just one increment above the minimal level tested.

The partial effectiveness following subcutaneous administration indicates a systemic action by dimethoate similar to that observed [6] for Bayer L 13/59. The species-selective action was also evident in these tests, but the general removal efficacies tended to be lower than those resulting from corresponding dosages administered via stomach tube. Higher dose rates by the subcutaneous route did not appear feasible

* Dipterex or Dylox, Chemagro Corporation, New York, N.Y.

because of the relatively large volume of solution required and consequently were not tested.

Increased efficacy on bots following administration by incorporation in the feed was reported [4, 5] in the initial trials on dimethoate; this also was a consistent observation throughout the present study. This has also been recorded for Bayer L 13/59 and DDVP by other workers.[6] Thus, this feature appears to be characteristic for the group of organic phosphates in the horse. A plausible explanation offered [6] is that the slow consumption of the drug in the feed provides efficacious levels for longer periods than those following stomach tube administration.

To obtain high-level removal (90%) of all bots (including the third instar *G. intestinalis*) by administration in the feed, three daily doses at the rate of 20 mg./kg. were required. This number of doses would appear to limit the practical application of present preparations of dimethoate, because the offensive odor and unpalatable nature make the consumption of medicated feed generally difficult and unpredictable. Individual variation of the animals rather than the dosage level was largely responsible for the varied acceptance of the medicated feed. Some horses were quite reluctant to eat a grain ration containing the drug at the rate of 10 mg./kg., whereas others readily consumed twice this dosage.

Selective removal of second instar bots was also quite evident in the feeding trials. The present tests did not determine the minimal effective dose regimen because the lowest dosage tested (3 doses at 10 mg./kg.) was still 100% effective on the second instars. Further investigation along these lines would offer the possibility of practical application for bot control by exploiting the selective removal of the second instars through the use of small doses at intervals during the bot transmission season.

The current practice of delaying treatment for bot removal until after transmission has ceased in the late fall cannot be regarded as a good therapeutic practice. It does afford perhaps the maximum measure of bot control that any one treatment could achieve, because the bots have accumulated to their maximum number by this time; however, it does ignore the harmful effects on the animal during the long period of time in which the bots are accumulating. This one-treatment practice has evolved in part from and has been conditioned by the traditional drug, carbon disulfide, which is difficult to administer and generally hazardous to use because of its toxic nature. Thus, an effective agent easily administered at intervals during the bot transmission season would afford a good measure of improvement in the therapy for bots of the horse.

Although the anthelmintic activity of dimethoate was quite limited in these tests, it was enhanced by administration in the feed; this has been generally detrimental to the action of other anthelmintics. The most apparent improvement by feed administration was efficacy against *Parascaris equorum*, which was refractory to single doses but partially (70%) removed by three 20 mg./kg. doses in the feed. Limited evidence also indicated that the foregoing treatment in the feed was more effective against adult female *Oxyuris equi* than the single doses via stomach tube. In contrast, using reductions of strongyle egg counts on feces after treatment as an index of possible antistrongyle activity, there were no consistent and striking changes even in the feeding trials. Softening of feces, which generally resulted after treatment, has to be taken into account as a dilution factor in the interpretation of the lower strongyle egg counts after treatment.

The toxic reactions observed for dimethoate in the present trials were similar to those described by others [6] for organic phosphates in the horse. Effects on the gastrointestinal tract were dominant, and a transient softening of the feces was the most common evidence of a mild toxic reaction. This was seen in horses treated with single doses at 60 mg./kg. by stomach tube and with multiple daily 20 mg./kg. doses in the feed. A severe toxic reaction was produced in the horse given the highest (80 mg./kg.) single dose in the tests. The reaction was characterized by severe gastrointestinal disturbance and was accompanied by generalized weakness and depression.

References

[1] Anonymous: Bibliography of Korlan and Trolene. The Dow Chemical Co., Midland, Mich., 1959.

[2] Bolle, W. R.: Controlling Larvae of *Gastrophilus intestinalis* with Neguvon. Vet.-medizinische Nachr., 4, (1957): 3–12.

[3] Drudge, J. H., Leland, S. E., Jr., Wyant, Z. N., Elam, G. W., Smith, C. E., and Dale, E.: Critical Tests with Piperazine-Carbon Disulfide Complex (Parvex) Against Parasites of the Horse. Am. J. Vet. Res., 18, (Oct., 1957): 792–797.

[4] Drudge, J. H., Leland, S. E., Jr., Wyant, Z. N., and Elam, G. W.: Critical Tests on the Organic Phosphate

Insecticide, Dimethoate, Against Bots in the Horse. 71st Ann. Report of the Director, Kentucky Agric. Exper. Sta., (1958): 54–55.

[5] Drudge, J. H., Leland, S. E., Jr., Wyant, Z. N., and Elam, G. W.: Critical Tests on the Organic Phosphate Insecticide, Dimethoate, Against Gastrophilus Species in the Horse. J. Parasitol., 45, (1959): 56.

[6] Drummond, R. O., Jackson, J. B., Glass, E. E., and Moore, B. Systemic Insecticides for the Control of Gastrophilus Bots in Horses. Agric. Chemicals, 14, (Dec., 1959): 41–43.

[7] Graham, O. H., Kraemer, P., and Osorio, A.: Control del Torsalo (*Dermatobia hominis*, L., Jr.) con Insecticidas Organicos Fosforados de Accion Sistemica. Turrialba, 8, (1958): 153–157.

[8] Hewitt, R., Emro, J., Entwistle, J., Pankavitch, J., Thorson, R., Wallace, W., and Waletzky, E.: Carbonyl Alkyl Phosphorodithioates as Chemotherapeutic Agents: Effects of Dimethoate Against Grubs in Cattle. J. Econ. Ent., 51, (1958): 445–450.

[9] Johnson, W. P.: The Effect of Dimethoate on *Dermatobia hominis* in Cattle. Am. J. Vet. Res., 21, (Nov., 1960): 1046–1048.

[10] Michel, H. O.: An Electrometric Method for the Determination of Red Blood Cell and Plasma Cholinesterase Activity. J. Lab. & Clin. Med., 34, (1949): 1564.

[11] Peterson, H. O., Cobbett, N. G., and Meleney, W. P.: Treatment of *Oestrus ovis* with Dimethoate. Vet. Med., 54, (1959): 377–383.

[12] Todd, A. C., and Brown, R. G., Jr.: Critical Tests with Toluene for Ascarids and Bots in Horses. Am. J. Vet. Res., 13, (April, 1952): 198–200.

SUMMARIO IN INTERLINGUA

Tests Critic, con le Insecticida a Phosphato Organic "Dimethoato," Contra *Gastrophilus spp.* in le Cavallo, con Observationes in Re Su Action Anthelmintic

Le suppression de larvas de gastrophilo e le activitate anthelmintic del phosphato organic "dimethoato" esseva evalutate in un serie de 21 tests critic in cavallos.

Action selective contra *Gastrophilus nasalis* esseva evidente. In tests con administrationes unic per medio de tubos gastric, le complete suppression de *G. nasalis* in tanto le secunde como etiam le tertie instar esseva jam effectuate con 20 mg/kg. Per contrasto con isto, un solmente restringite action contra *G. intestinalis* in le tertie instar esseva notate post doses unic augmentate usque al magnitude de 80 mg/kg, le qual es toxic pro cavallos. *G. intestinalis* in le secunde instar esseva plus susceptibile. In summa, 80% de istos esseva eliminate per doses unic de inter 20 e 80 mg/kg.

Le administration del agente in le forrage augmentava marcatemente le efficacia de illo contra le larvas de gestrophilo, e tres tal doses diurne de 20 mg/kg eliminava plus que 90% de ambe le mentionate species in tanto le tertie como etiam le secunde instar. Tamen, variationes individual in le maniera in que le animales acceptava le medicate forrage rendeva iste modo de administration generalmente difficile e inpredicible, e leve formas de reaction toxic accompaniava le tractamento.

Le activitate anthelmintic de dimethoato esseva limitate, sed—a generalmente parlar—illo esseva promovite per un administration con le forrage. *Parascaris equorum* esseva refractori contra doses unic, sed 70% de illo (al media) esseva eliminate per tres doses diurne de 20 mg/kg in le forrage. Restringite datos indica que etiam adultos de *Oxyuris equi* esseva eliminate plus efficacemente per multiple doses diurne in le forrage. Le alterationes post-therapeutic in le numerationes de ovos de strongylo esseva multo variabile. Interpretate como indice de activate anti strongylo, ille numerationes revelava nulle uniforme o frappante reductiones mesmo post administration in le forrage.

Preliminary Findings Concerning the Formalin Inactivation of Herpes B Virus

James E. Prier, D.V.M., Ph.D., and Normand R. Goulet, Ph.D.

SUMMARY

Inactivated herpes B virus was demonstrated to be capable of inducing protection in rabbits. Such protection was not associated with high levels of neutralizing antibody. Safety tests on treated suspensions showed that, unless inactivation was carefully controlled, residual amounts of virus persisted for periods up to 21 days after chemical treatment. Preliminary work suggests the existence of strain differences among B virus isolates.

INTRODUCTION

Infection with herpes B virus is a risk in people using monkeys in biologic work or in persons having contact with Old World primates.[1, 4, 7, 8, 10-12]

The two points of logical interference in the epidemiology of herpes B virus infection are to render either man or the monkey immune to the disease. There is no evidence available to indicate whether the monkey is capable of harboring and disseminating virus after immunization with parenterally administered killed antigen. Also, vaccination of monkeys would require the supervised treatment of large groups of animals at or near their natural habitat.

Experimental immunizing agents for herpes B virus have been reported.[3, 5] These authors produced formalin-inactivated vaccines that were capable of protecting rabbits against herpes B virus challenge. The present report confirms some of their results, and extends certain aspects of experimental immunizing agents for herpes B virus with particular reference to inactivation, potency, and safety.

Received for publication Oct. 31, 1960.

From the School of Veterinary Medicine, University of Pennsylvania, Philadelphia (Prier) and the Biological Development Department, Merck Sharp & Dohme, West Point, Pa. (Goulet). Dr. Goulet is now with the National Drug Co., Philadelphia, Pa.

The technical assistance of James M. Silliman and Robert W. LeBeau in conducting the experimental work is acknowledged. The authors also thank Dr. Albert B. Sabin, Department of Pediatrics, University of Cincinnati College of Medicine, for his advice and cooperation in discussing related problems and data, many of which are not covered by this report.

Materials and Methods

Virus Strains.—Three strains of herpes B virus were used in these studies. The O'Hara strain was obtained from Dr. F. P. Nagler, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario, as first passage cell cultured virus that was originally isolated from a human being with a fatal case of herpes B virus infection. Second and third rabbit kidney cell culture passages were made in this laboratory on June 17, 1958, and July 7, 1958, and the latter was used for seed virus for experimental purposes. The Sabin strain was obtained from Dr. Albert Sabin, University of Cincinnati, in February, 1958, as a single passage of virus originating from a human being with a fatal case of herpes B virus encephalitis. The strain was maintained in this laboratory by serial passage in rabbit kidney cell culture. Preliminary studies were done using a strain of virus from Dr. J. G. Crawford, of the Biological Laboratories of Chas. Pfizer & Co., Inc. This agent, designated as herpes B virus strain 3088, was recovered from monkey kidney cultures, and was received as the 3rd monkey kidney culture passage. This strain of virus has some characeristics typical of herpes B virus, but has less virulence for rabbits.

Rabbit Kidney Cell Cultures.—Roller tubes and 1-liter Blake bottles were prepared with kidney cells obtained from 3- to 4-week-old rabbits. Cells were removed from kidney tissue with 0.25% trypsin (Difco 1:250) for 2 hours at room temperature in a 500-ml. fluted flask. The cells were harvested every 30 minutes by pouring off the supernatant fluid and renewing the volume with fresh trypsin. Harvested material was pooled at the completion of the trypsinizing runs. Cells were counted in a hemacytometer, and 20 million cells were placed in each 1-liter Blake bottle. The volume of nutrient fluid was 100 ml. per Blake bottle. Medium 199 with 10% calf serum containing 100 units of penicillin and 100 μg. of streptomycin per milliliter of medium was used. After three days of growth at 37 C., a change of medium was made to medium 199 containing 5% calf serum.

On the 5th day, a final change was made to medium 199 containing 2% calf serum and cell cultures employed for virus studies. The procedures employed for the growth and maintenance of monkey kidney cultures were essentially similar to the methods reported by Youngner,[18] but overnight cold trypsinization was employed in all cases. The medium employed for growth and maintenance of these cultures was 199 and 2% calf serum.

Virus Suspensions.—Approximately 1.0 ml. of stock virus with an average titer of $10^{6.7}$ $TCID_{50}$/0.25 ml. was added to each Blake bottle containing rabbit tissue. After 48 to 72 hours of growth at 37 C., the cells were completely destroyed by the virus, and the fluid was harvested and stored in 9-liter bottles at 4 C. Prior to inactivation, the virus fluids were filtered through a coarse fritted glass candle under 2 to 4 lb. of positive pressure.

Titration of Viruses.—The quantity of virus was estimated by determining the limiting dilution which could initiate infection in 50% of the cell cultures inoculated. Serial half-log dilutions of virus were prepared and 0.25-ml. aliquants of each dilution were added to four tubes of cell culture. The cultures were incubated at 37 C. and examined each day for seven days for cytologic changes characteristic of herpes B virus infection.

Inactivation.—The details of the study were obtained with the Sabin and O'Hara strains, and one lot of vaccine was prepared from strain 3088 for cross-immunization studies. Varying concentrations of formalin from 1:400 through 1:4,000 were used to inactivate herpes B virus suspensions. Temperatures and times of inactivation also varied. The total amount of formalin required was added in a volume of Hanks' balanced salt solution that was 10% of the total volume inactivated. Samples were removed from the inactivating material for virus assay at different times, depending on the inactivation method. Samples for virus assay were neutralized with sodium bisulfite and dialyzed against 20 volumes of phosphate-buffered saline solution (pH 7.2). Solutions of β-propiolactone were used to inactivate some vaccines. When used alone, 800 mg. per liter was used; and in combination with formalin, 400 mg. per liter were added. Vaccine virus was in contact with the inactivating agents for 96 hours before rabbit inoculation.

Safety Tests—Rabbit Inoculation.—Each rabbit was given 10 ml. of vaccine by the following routes: 4.5 ml. in each of two sites subcutaneously, and 0.5 ml. in each of two sites intradermally. Animals were observed for 21 days for signs of local or systemic disease.

Serum Neutralization.—Serum neutralization tests were performed by mixing equal portions of virus (approximately 100 $TCID_{50}$) with dilutions of serum and incubating the mixtures for two hours at 37 C. This was followed by overnight incubation at 4 C. before testing for neutralization in rabbit kidney tissue cultures.

Cell Culture Tests.—A total of 200 ml. of each vaccine was placed on cultures of rabbit kidney. Forty milliliters was placed in each of five 1-liter bottles of monolayer culture, and 60 ml. of medium was added to each bottle. After seven and 14 days of incubation, fluids were removed from the bottles and put in roller tubes containing rabbit kidney cells. One milliliter of fluid was placed in each tube. After 21 days of incubation, the original cell sheets were scraped into the medium, and 1 ml. of cell suspension was placed in each of 20 roller tubes of rabbit kidney monolayer cell culture. The tubes, employed in subpassages, were read daily for seven days.

Potency Tests.—The antigenic activity of vaccines was tested in rabbits. An antigen dose-response procedure was used, including the following doses of vaccine; undiluted, 1:5, 1:25, and 1:125. Two injections of 1 ml. each were given 14 days apart. Fourteen days after the second injection, immunity of all animals, including unvaccinated controls, was challenged with 1,000 rabbit LD_{50} of herpes B virus. The total 1-ml. dose was injected in equal amounts at five separate intradermal sites. Potency was expressed as the dose of vaccine required to protect 50% of the experimental animals.

Antiserum Production.—Herpes B virus grown in monkey kidney cells was inactivated with 1:4,000 formaldehyde for 48 hours at 37 C. The material was then stored at 5 C. without neutralization of the formaldehyde. Five milliliters of material was injected intraperitoneally into rabbits three times per week for a total of four weeks. Blood samples were obtained one week after the last injection, and serum was removed and stored at −17 C.

Results

Inactivation.—It had been demonstrated previously in this laboratory* and in others[3,5] that prolonged exposure of formalin-treated virus at 36 C. was associated with a loss in antigenicity. Accordingly, the majority of inactivations were conducted at 4 C. It soon became apparent that a major problem in the inactivation procedure was the presence of residual live virus for varying periods.

In Fig. 1, curve A shows the effect of adding 1:1,000 formalin to a herpes B virus suspension at 4 C. Although most of the virus had been inactivated, there was a definite "tailing effect" and consequent persistence of a low concentration of active virus. Curve C shows the results of doubling the concentration of formalin at eight hours of inactivation. Complete inactivation resulted, but this material

* Prier, J. E.: Unpublished data, University of Pennsylvania School of Veterinary Medicine, Philadelphia, 1958.

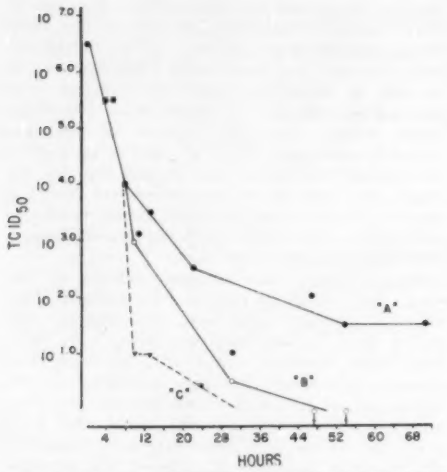


Fig. 1—Effects of formalin and heat on herpes B virus preparations.

also was devoid of antigenicity when subsequently tested in rabbits.

Curve B illustrates the decreased inactivation time achieved by introducing a 22-hour period of 37 C. inactivation eight hours after formalin addition.

The rate of inactivation of untreated herpes B virus suspension held at 5 C. was determined. The effect of storage in serum-free medium on herpes B virus viability is shown (Fig. 2). These fluids contained polio virus, and the herpes B virus was a natural contaminant of the cell substrate. The phenomenon of persistence at low virus concentration also was demonstrated,

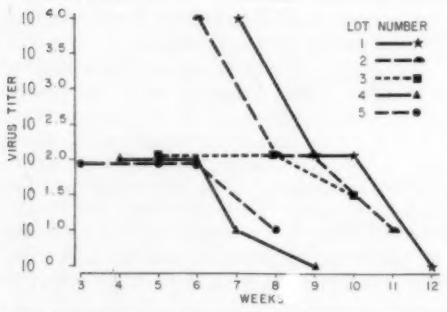


Fig. 2—Inactivation of untreated herpes B virus at 5 C. in serum-free medium (tissue culture titrations).

TABLE 1—Potency of Herpes B Virus Vaccines in Rabbits

Vaccine dilution	Survival ratios following challenge with herpes B virus*					
	5	8a	9c	9d	9e	9f
Undiluted	17/19	6/6	2/4	3/4	2/3	3/5
1:5	N.D.**	4/6	0/4	3/4	0/2	1/5
1:25	N.D.	0/6	0/3	1/4	0/4	0/5
1:125	N.D.	0/4	N.D.	N.D.	N.D.	0/5
None	0/10	0/6	0/4	0/4	0/4	0/5

Vaccines 5 and 8a = 1:1,000 formalin at 4 C., tested after 96 hours; 9c = 1:1,000 formalin at 4 C. + 400 mg./liter β-propiolactone, after 10 hours, and tested after 96 hours; 9d = 1:1,000 formalin for 24 hours, at 37 C. with subsequent storage at 4 C.; 9e = 1:1,000 formalin at room temperature (controlled temperature 78 F.) for 24 hours with subsequent storage at 4 C.; and 9f = β-propiolactone (800 mg./liter), 40 C., tested after 96 hours.

* Survival/total tested. ** N.D. = not done.

whereas materials with higher titers lost virulence rather rapidly.

Safety.—The preparation of a vaccine free of residual live virus was crucial. The safety testing of these vaccines in tissue culture indicated that live virus was present for longer than 14 days when cold-inactivation was employed. Such tests were consistently negative after 28 days of inactivation at 4 C.

Potency.—Determination of the immune response was based on survival of immunized rabbits following a direct live-virus challenge which regularly resulted in 100% mortality of the controls. An antigen dose-response method was considered a more sensitive method of demonstrating antigenicity than the use of single or multiple injections of undiluted vaccine.

The protection afforded rabbits with some of the vaccines tested is shown (Table 1). These data leave no doubt that a herpes B virus vaccine can induce resistance in rabbits.

Thus far, resistance to herpes B virus paralysis and death has been associated with antibody levels as low as 1:4 and 1:8. Low antibody levels in laboratory animals after injection of formalin-inactivated herpes B virus were reported also.[2] The data (Fig. 3) indicate that these levels are associated with protection of rabbits. The protective levels were obtained with undiluted vaccine and in some cases with vaccine that had been diluted 1:5. The data do not establish whether higher antibody levels after challenge are the result of giving live virus or whether the antibody response is merely due to a third inoculation of antigen, per se.

The results obtained when rabbits were immunized with the strain 3088 agent inactivated with 1:1,000 formalin at 4 C. are shown (Table 2). This vaccine was prepared by the same methods and with approximately the same results as obtained with the other two strains of virus. Control rabbits employed in this experiment were given vaccine 9e (Table 1; 1:1,000 formalin for 24 hours at room temperature with subsequent 4 C. storage) which included an adjuvant (Arlacel A-Drakeol). Their immunity was challenged with Sabin strain virus.

In tissue culture cross-neutralization tests, a "one-way cross" was demonstrated between the Sabin strain and the strain 3088 agent. It can be seen (Table 3) that neutralization titers were obtained in the homologous systems. However, Sabin strain antiserums failed to neutralize the strain 3088 virus, whereas 3088 antiserums neutralized the Sabin strain of herpes B virus.

TABLE 2—Potency of Strain 3088 Herpes B Virus in Rabbits

Vaccine dilution	Survival ratio after challenge with Sabin strain*	
	Strain 3088 herpes B virus	Sabin strain herpes B virus vaccine**
Undiluted	4/4	4/4
1:5	2/4	1/4
1:25	2/4	0/4

* Survival/total tested. ** Vaccine 9e (Table 1) plus Arlacel A—Drakeol.

Discussion

It is clear from these studies and others [3] that antigenic material capable of protecting rabbits from direct herpes B virus challenge can be prepared by tissue culture. That low levels of antibody were associated with protection of rabbits also was demonstrated.

The data for the 3088 strain, although preliminary and incomplete at present, indicate that strain differences, heretofore unrecognized, may be involved, or that agents having close antigenic similarity to herpes B virus may occur in monkeys. The lesions obtained after intradermal inoculation of rabbits with the 3088 strain were not entirely characteristic of herpes B virus lesions. Additional information will have to be obtained to determine if rabbits so inoculated are as susceptible as those inoculated with the Sabin or O'Hara strains of herpes B virus. The question now raised is whether a vaccine strain could be selected on the basis of comparative antigenicity and virulence, and indeed, whether a polyvalent vaccine may be necessary for adequate protection against the disease.

The problem of safety of the vaccines is an important consideration. The question of man's relative sensitivity to infection with herpes B virus has been raised.[6] The only tests available at present are rabbit injection and cell culture infection. It is known that both living systems are equally susceptible to untreated virus, but formalin-treated, partially inactivated virus is more virulent for cell culture than for rabbits. It, therefore, becomes of interest whether man is less or more susceptible than other biologic systems.

Many other factors, including the persistence of immunity and the number and spacing of injections, must be known before a rational approach to immunization can be determined.

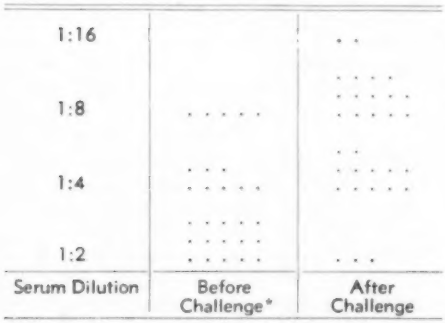


Fig. 3—Effect challenge virus on circulating antibody levels in surviving immunized rabbits. *Serum from 3 rabbits not tested.

TABLE 3—Cross-Neutralization Tests in Tissue Culture with Herpes B Virus, Sabin and 3088 Strains

Virus (100 $TCID_{50}$)	Antiserum	Reciprocal of antiserum dilution
Sabin B-II	B-II	32
Sabin B-II	3088	32
3088	3088	16
3088	B-II	0

References

[1] Breen, G. E., Lamb, S. G., and Otaki, A. T.: Monkey-Bite Encephalomyelitis, Report of a Case—with Recovery. Brit. Med. J., 2, (1958): 22–23.

[2] Burnet, F. M., Lush, D., and Jackson, A. V.: The Relationship of Herpes and B Viruses: Immunological and Epidemiological Considerations. Austral. J. Exptl. Biol. & Med., 16, (1938): 41–51.

[3] Hull, Robert N., and Nash, J. C.: Immunization Against B Virus Infection. I. Preparation of an Experiment Vaccine. Am. J. Hyg., 71, (1960): 15–28.

[4] Hummeler, K., Davidson, W. L., Henle, W., La-Bocetta, A. A., and Ruch, H. G.: Encephalomyelitis Due to Infection with *Herpesvirus simiae* (Herpes B Virus), A Report of Two Fatal Laboratory-Acquired Cases. New England J. Med., 261, (1959): 64–68.

[5] MacLeod, D. R. E., Shimada, F. T., and Walcroft, N. J.: Experimental Immunization Against B Virus. Ann. New York Acad. Sci., 85, (1960): 980.

[6] Melnick, J. L., Benyesh-Melnick, M., and Brennan, J. C.: Studies on Live Poliovirus Vaccine (Its Neurotropic Activity in Monkeys and Its Increased Neurovirulence After Multiplication in Vaccinated Children). J.Am.M.A., 171, (1959): 1165–1172.

[7] Nagler, F. P., and Klotz, M.: A Fatal B Virus Infection in a Person Subject to Recurrent *Herpes labialis*. Canad. M. A. J., 79, (1958): 743–745.

[8] Pierce, E. C., Pierce, J. D., and Hull, R. N.: B-Virus: Its Current Significance. Description and Diagnosis of a Fatal Human Infection. Am. J. Hyg., 68, (1958): 242–250.

[9] Reed, L. J., and Muench, N.: Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., 27, (1938): 493–497.

[10] Sabin, A. B.: Studies on the B Virus. I. The Immunological Identity of a Virus Isolated from a Human Case of Ascending Myelitis Associated with Visceral Necrosis. Brit. J. Exptl. Path., 15, (1934): 242–268.

[11] Sabin, A. B.: Fatal B Virus Encephalomyelitis in Physician Working with Monkeys. J. Clin. Invest., 28, (1949): 808.

[12] Sabin, A. B., and Wright, A. M.: Acute Ascending Myelitis Following Monkey Bite, with Isolation of Virus Capable of Reproducing Disease. J. Exptl. Med., 59, (1934): 115–136.

[13] Youngner, J. S.: Monolayer Tissue Cultures. I. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells. Proc. Soc. Exptl. Biol. & Med., 85, (1954): 202–205.

SUMMARIO IN INTERLINGUA

Constatationes Preliminari Concernente le Inactivation de Virus B de Herpes

Esseva demonstrate que inactivate virus B de herpes es capace a inducer protection in conilios. Iste protection non esseva associate con alte nivellos de anticorpore neutralisante. Tests de securitate con tractate suspensiones revelava que si le inactivation non es cautemente controlate, quantitates residue de virus persiste durante periodos de usque a 21 dies post le tractamento chimic. Studios preliminari suggere le existentia de differentias de racia inter isolatos de virus B.

The Leukotaxic Properties of Uterine Exudates as Related to the Endocrine-Controlled Uterine Defense Mechanism

H. W. Hawk, Ph.D.; G. D. Turner, B.S.; J. F. Sykes, Ph.D.

SUMMARY

Rabbits were killed one, two, and four hours after experimental inoculation with *Escherichia coli*. The influx of leukocytes into uterine lumens was quickest and most intense in estrous rabbits and progressively less in ovariectomized and pseudopregnant ones. However, cell-free uterine flushings from the pseudopregnant rabbits evoked the greatest leukocytic response in rabbit skin and in rabbit uteri and increased skin capillary permeability to the greatest degree. The results suggested that hormonal control of the uterine defenses was accomplished primarily through mechanisms other than the capacity of uterine contents to attract leukocytes or increase capillary permeability.

Further evidence was obtained that progestins inhibit the passage of leukocytes through uterine tissues.

INTRODUCTION

The ovarian status of an animal influences the uterine defense mechanisms during the first few hours of induced infection.[2,5] Hormonal effects are shown by variation in the time required for development of the leukocytic response and time required for clearance of bacteria from the uterine lumen. Progesterone delays the leukocytic response markedly; estradiol stimulates the response.[6]

The mechanisms by which ovarian hormones influence the leukocytic response are not clear. One possibility could be hormonal control of the capacity of the uterine contents to attract leukocytes.[3] Another possibility might be hormonal control of the ability of uterine contents to change capillary permeability during the early stages of inflammation.

This report concerns investigations on the leukotaxic properties of uterine exudates collected during the early stages of induced infection, the ability of these exudates to increase capillary permeability, and the relationship of these factors to hormonal control of the leukocytic response.

Experiment 1—The Effect of Uterine Exudates in Skin

Methods.—Estrous, pseudopregnant, and ovariectomized rabbits were used.[6] Each rabbit was inoculated in one ligated uterine horn with approximately 200 million *Escherichia coli* cells in 0.2 ml. of 0.87% NaCl solution. Rabbits were killed one, two, or four hours after inoculation. Each horn of each rabbit was flushed with sterile saline solution to a volume of 0.35 ml. In some instances, the uninoculated horn was flushed with 0.2 ml. of the same *Esch. coli* suspension used in the inoculum, followed by a small amount of saline solution. Specimens from each horn were fixed in Bouin's solution.

The flushings were centrifuged at 100g for 10 minutes to remove leukocytes and again at 2,500g for 40 minutes, which removed almost all of the remaining *Esch. coli*.

Sediment from the centrifugations was used to estimate the number of leukocytes flushed from each uterine horn and to determine grossly the extent to which *Esch. coli* had been cleared from the uterine lumens.

The depilated abdominal skin of rabbits in the follicular phase of the estrous cycle was used as test sites for the uterine flushings. A 27-gauge hypodermic needle was passed through the skin for about 20 mm. and 0.1 ml. of each flushing was deposited intradermally. Other injections into each recipient rabbit also included the centrifuged flushings from uninoculated horns and sterile saline solution. The injection sites were always spaced at least 30 mm. apart.

Each recipient rabbit was given intravenously 70 mg. of trypan blue dye in saline solution. The degree of dye accumulation over each skin injection site was graded on a scale from 0 to 8 at several time intervals after injection; 0 indicated coloration no greater than that of uninjected skin, 8 indicated extremely intense coloration. In a preliminary trial, there was no suggestion that dye exudation into the skin influenced the leukocytic infiltration of the area greatly.

Received for publication July 5, 1960.

From the Dairy Cattle Research Branch, Animal Husbandry Research Division, Agricultural Research Service, USDA, Beltsville, Md.

Two hours after the skin injections, the recipient rabbits were killed and each injection site was excised and fixed in Bouin's solution. The excised skin specimen included the panniculus carnosus and underlying fascia. Specimens were usually slightly over 1 cm. in diameter.

Skin specimens were sectioned at 9 μ and stained with hematoxylin and eosin. Microscopic examination was made of at least 16 sections, covering a width of about 3 mm. through the center of each specimen.

The intensity of the leukocytic infiltration in skin and in uterine tissues of exudate-donor rabbits was graded on a scale from 0 to 8. A lack of response was 0, a doubtful response was 1, a definite response but limited in intensity was 2, etc., with 8 indicating a severe response. With the uterine horns, corrections were made in the indexes of leukocytic infiltration to account for variation in length. The indexes thus reflect total leukocytic infiltration of each uterine horn.

Results.—Mean numerical scores are listed for leukocytic infiltration of the inoculated exudate-donor uteri, leukocytic infiltration of the skin exudate-injection sites, and dye coloration of the skin injection sites (Table 1). Also given are the mean numbers of leukocytes flushed from uteri of rabbits killed four hours postinoculation (footnote, Table 1).

and ovariectomized rabbits over the three time intervals differed little in leukotaxic properties. Nevertheless, the leukocytic response *in uteri* of these rabbits differed markedly. Statistically, the leukocytic response to uterine inoculation in exudate-donor rabbits was independent of the leukotaxic properties of the uterine contents ($P<0.01$).

Saline solution alone, when injected into the skin, caused a slight leukocytic response in some rabbits, possibly the result of injury to the skin tissues. There was a more intense response to flushings from uninoculated horns, the greatest being to those from pseudopregnant rabbits. Thus, "leukotaxic" substances normally present in the uterus probably accounted largely for the greater leukotaxic properties of inflammatory exudates from pseudopregnant rabbits.

Flushings from uninoculated horns flushed with *Esch. coli* suspensions evoked a greater response than did flushings from similar horns flushed with sterile saline solution, suggesting that noncellular leukotaxic substances were present in the uterine inoculums. The greatest responses were

TABLE 1—The Leukocytic Response of Rabbits to Uterine Inoculation with Escherichia coli and the Response in Skin to Exudates from the Inoculated Uteri

Leukocytic infiltration of exudate-donor uteri				Skin response to uterine exudates					
				Estrous		Pseudopregnant		Ovariectomized	
Hours of inflammation	Estrous rabbits	Pseudopregnant rabbits	Ovariectomized rabbits	Leukocytic infiltration	Dye coloration	Leukocytic infiltration	Dye coloration	Leukocytic infiltration	Dye coloration
1	1.0(6)*	0.4(6)	0.0(4)	3.9	1.4	5.2	5.8	5.2	0.8
2	5.6(6)	3.9(6)	2.0(4)	5.4	3.9	6.3	4.7	4.8	1.2
4	7.3(4)	5.8(4)	5.0(2)	4.7	4.3	7.7	6.7	4.5	1.5

Mean numbers of leukocytes flushed from uterine lumens at four hours: estrous rabbits, 256,000,000; pseudopregnant rabbits, 12,000,000; ovariectomized rabbits, 38,000,000. (Numbers of leukocytes in one- and two-hour flushings were negligible.)

Clearance of *Esch. coli* from uterine lumens: in estrous and ovariectomized rabbits, slight at one and two hours, nearly complete at four hours; in pseudopregnant rabbits, slight at each time interval.

* Values represent degrees of leukocytic infiltration or dye coloration graded on a scale from 0 to 8. Figures in parentheses indicate number of rabbits in the group.

The injection of uterine inflammatory exudates into skin of other rabbits evoked a marked leukocytic response. However, there was no indication that ovarian hormones influenced the leukocytic response by controlling the amount of leukotaxic substances in the uterine lumens. In general, exudates from the pseudopregnant donors evoked the greatest leukocytic response in skin, but the influx of leukocytes into the uterine lumens was slowest in these rabbits. Exudates collected from estrous

evoked by inflammatory exudates, suggesting that leukotaxic substances were being produced during the inflammatory process, either by the uterine tissues, *Esch. coli*, or both. It was readily apparent that the leukotaxic substances in the uterine flushings did not come from one source only.

Capillary permeability, as measured by blue coloration of the skin, was changed markedly by some of the exudates (Table 1). Exudates obtained at each time interval from pseudopregnant rabbits caused

intense blue coloration over an area 1 to 2 cm. in diameter. One-hour estrous rabbit exudates induced only limited dye diffusion, but two- and four-hour exudates caused considerable coloration. Exudates from ovariectomized rabbits, even those obtained four hours after uterine inoculation, had little effect on capillary permeability.

The uterine contents of estrous and pseudopregnant rabbits had an earlier and greater capacity to increase capillary permeability than did exudates from ovariectomized rabbits. Diapedesis of leukocytes also occurred slightly earlier in estrous and pseudopregnant rabbits. Beyond this possible cause and effect, there was no evidence that endocrine control of the leukocytic response was achieved through an effect on the ability of the uterine contents to change capillary permeability.

The substances which increased capillary permeability were apparently produced in the uterus. Neither saline solution nor supernatant fluids of *Esch. coli* suspensions affected capillary permeability. Flushings from uninoculated horns of pseudopregnant rabbits increased capillary permeability markedly; those from estrous or ovariectomized rabbits did not.

Flushings from inoculated horns of estrous and pseudopregnant rabbits might contain disproportionate amounts of two substances or groups of substances: those leukotaxic in skin only and those active in both the skin and uterus. If so, exudates of pseudopregnant rabbits might attract more leukocytes when injected into skin, even though exudates of estrous rabbits might draw more leukocytes into the uterus during inflammation. Leukotaxic properties of exudates from estrous and pseudopregnant rabbits were therefore compared in the uterus.

Experiment 2—Leukotaxic Properties of Exudates When Tested in Uterine Lumens

Methods.—Exudates were collected from two pairs of estrous and pseudopregnant rabbits one hour after uterine inoculation and from six pairs two hours after inoculation. It was assumed that any leukotaxic substances which caused differences in the leukocytic response between two and four hours would likely be present in two hours.

Uterine flushings were handled as described previously. Flushings from inoculated horns only were used. Each pair of exudates was tested in the same rabbit, the entire exudate from one inoculated horn being injected into one ligated horn of the recipient rabbit. Exudates were tested in both estrous and pseudopregnant rabbit uteri. Recipient rabbits were killed four hours after exudate injection, and the degree of leukocytic infiltration of the uterine tissues was rated numerically.

Results.—Exudates from both estrous and pseudopregnant rabbits evoked a moderate leukocytic response in the uterus. In general, exudates from pseudopregnant rabbits were slightly more leukotaxic than were those from estrous ones (Table 2),

TABLE 2—The Leukocytic Response to Uterine Inoculation of Rabbits with Escherichia coli and the Response in Uteri to Cell-Free Exudates from the Inoculated Uterine Horns

Hours of inflammation	Leukocytic infiltration of exudate-donor uteri: Estrous rabbits	Leukocytic infiltration of exudate-donor uteri: Pseudopregnant rabbits	Response to estrous rabbit exudates	Response to pseudopregnant rabbit exudates
1	1.0(2)*	0.7(2)	3.2	4.0
2	4.3(6)	2.7(6)	4.5	4.8

* Number of rabbits in the group is shown in parentheses.

whether the exudates were assayed in estrous or pseudopregnant rabbits.

As in the previous experiment, uteri of the estrous exudate donors were infiltrated the most heavily with polymorphonuclear leukocytes (Table 2). The response to uterine inoculation in exudate-donors of the two endocrine states was independent of the leukotaxic properties of the uterine contents ($P<0.02$, as measured statistically).

Endocrine Effects on the Leukocytic Response.—The pattern of leukocytic response in exudate-donors of the three endocrine states confirmed previous observations that ovarian hormones influence two phases of the response: (1) the migration of leukocytes from blood vessels[6] and (2) the passage of leukocytes into the uterine lumen.[3,6]

It appears that both estrogen and progestin (estrogen to a greater degree) promote conditions favorable to a rapid migration of leukocytes into the uterine tissues after experimental inoculation. Both estrous and pseudopregnant rabbits had greater initial tissue leukocytosis than did ovariectomized rabbits (one- and two-hour values for leukocytic infiltration of exudate donors, Table 1).

The initial tissue leukocytosis in pseudopregnant rabbits was intermediate between that in estrous and ovariectomized rabbits, but pseudopregnant rabbits had the fewest leukocytes in the uterine lumens at four hours. This suggests that the passage of

leukocytes through the uterine tissues was inhibited in pseudopregnant rabbits, presumably through some action of progestin. A direct effect of progestin on leukocytes cannot be ruled out, but it seems more likely that the hormone affects leukocytic migration in some other way. It may be relevant that in estrous and ovariectomized rabbits two thirds of the total endometrial water is extracellular, but in progesterone-influenced rabbits about one third is extracellular.[1] The relatively small extracellular water volume in pseudopregnant rabbits might impede leukocytic migration.

Discussion

It is not known whether the noncellular "leukotaxic" substances present in rabbit uteri were essential for initiation and maintenance of the leukocytic response. Probably the response was at least intensified by their presence. It was apparent, however, that ovarian hormones did not primarily influence the uterine defense through the gross amounts of these substances in the uterine lumens. Rather, the results suggest that ovarian hormones act through other tissues or mechanisms participating in the inflammatory response.

A variety of biologic substances have been shown to cause a leukocytic response when injected into animal tissues (*see* review by Harris [4]). It is therefore unlikely that the substances which evoked a leukocytic response in these rabbits were homogeneous in nature. Whether any or all of the "leukotaxic" substances had a specific attraction for leukocytes is not known.

The substances in the uterine extracts which evoked a leukocytic response and those which increased capillary permeability could have been identical to a limited degree only. All or nearly all of the permeability-affecting substances were produced in the uterus; a like proportion of the leukotaxic substances were not. Further, these two properties of the exudates were not always proportional (Table 1). Likewise, Spector and Story [7] found no correlation between the ability of mouse uterine extracts to cause leukocytic emigration and increase capillary permeability to trypan blue.

References

[1] Bitman, Joel, Cecil, Helene, Hawk, H. W., and Sykes, J. F.: Effect of Estrogen and Progesterone on Water and Electrolyte Content of Rabbit Uteri. Am. J. Physiol., 197, (1959): 93–98.

[2] Broome, A. W. J., Lamming, G. E., and Smith, Wendy: Studies on the Relationship Between Ovarian Hormones and Uterine Infection. IV. The Role of the Granulocyte System in Uterine Defense. J. Endocrinol., 19, (1959): 274–281.

[3] Broome, A. W., Winter, A. J., McNutt, S. H., and Casida, L. E.: Variations in Uterine Response to Experimental Infection Due to the Hormonal State of the Ovaries. II. The Mobilization of Leukocytes and Their Importance in Uterine Bactericidal Activity. Am. J. Vet. Res., 21, (July, 1960): 675–682.

[4] Harris, H.: Role of Chemotaxis in Inflammation. Physiol. Rev., 34, (1954): 529–562.

[5] Hawk, H. W.: The Influx of Leukocytes and Presence of Bactericidal Substances in Inoculated Uteri of Estrous and Pseudopregnant Rabbits. J. Anim. Sci., 17, (1958): 416–425.

[6] Hawk, H. W., Turner, G. D., and Sykes, J. F.: The Effect of Ovarian Hormones on the Uterine Defense Mechanism During the Early Stages of Induced Infection. Am. J. Vet. Res., 21, (July, 1960): 644–648.

[7] Spector, W. G., and Story, E.: A Factor in the Oestrogen-Treated Uterus Responsible for Leukocyte Emigration. J. Path. Bact., 75, (1958): 387–398.

SUMMARIO IN INTERLINGUA

Le Proprietates Leucotaxic de Exsudatos Uterin, in Relation al Endocrinmente Regulate Mechanismo de Defensa Uterin

Conilias esseva sacrificate un, duo, e quatro horas post le inoculation experimental de *Escherichia coli*. Le influxo de leucocytos ad in le lumines uterin esseva le plus rapide e le plus intense in conilias in estro e declinava progressivemente in ovariectomisatas e pseudopregnantes. Tamen, acellular eluitos uterin ab le conilias pseudopregnante evocava le plus grande responsa leucocytic in le pelle de conilios e in uteros de conilias e augmentava le permeabilitate capillari del pelle le plus marcatemente. Le resultatos suggere que le regulation hormonal del defensas uterin es complite primarimente per mechanismos altere que le capacitate del contento uterin de attraher leucocytos o de augmentar le permeabilitate capillari.

Esseva obtenite indicationes additional que progestinas inhibi le passage de leucocytos a transverso le tissu uterin.

Scientific Notes

Electron Microscopic Observations of a Mucoid Strain of Vibrio fetus

Henry James Werner, Ph.D.; Helen Levy, B.S.; Ben O. Spurlock

This investigation represents the first attempt at examining the cyto-architecture of Vibrio fetus by electron microscopy of ultrathin sections. Rhoades [13] used a shadow-casting technique in conjunction with the electron microscope. His work was concerned primarily with the external morphology of Vibrio fetus.

Materials and Methods

The cells were grown in a Roux flask in 250 ml. of Brucella broth (Albimi), and the inoculum was 4 ml. of a three-day culture in Brucella broth (containing 0.1% agar). Approximately 10% CO_2 was introduced into the flask atmosphere and the culture was incubated for four days at 37 C. The cells were harvested for ten minutes and then washed three times in physiologic saline solution buffered at pH 7.0.

The cells were fixed in 1.0% solution of osmium tetroxide in veronal-acetate buffer (pH 7.5) for one hour at 4 C. The osmolarity of the fixative was adjusted with sucrose. The cells were then rinsed in distilled water, dehydrated in a graded series of methanol, and embedded in a 1:3 mixture of methyl and n-butyl methacrylates. Polymerization was carried out at 45 C. Sections were cut with glass knives in an ultra-microtome and mounted on Formvar-coated grids.

Results and Discussion

Immediately apparent around the cells was a capsular residue (Fig. 1, A). Using Anthony's capsule stain,[4] a positive reaction for a mucoid capsule was obtained. Chapman [5] reported the cell membranes of certain bacteria as being composed of two dense membranes separated by a member of less density. Kellenberger [6] reported a multilayered cell membrane in Escherichia coli. Such is certainly not the case in V. fetus. We had to stain with heavy metals according to the technique described by Watson [15] in order to demonstrate the cell membrane which appeared even then as only a very delicate delimiting membrane (Fig. 1, B). The most obvious structures in the cytoplasm were the rather uniformly dense particles (Fig. 1, C). Grula [9] identified similar electron-opaque granules (volutin) composed of metaphosphate or another form of inorganic phosphate, some fat, and small amounts of protein. Hickman [7] and Vatter [14] both interpreted similar opaque, high-density particles as polyphosphates.

The cytoplasm of V. fetus, except for the presence of these particles, was found to be uniformly granular. At this point, one can merely speculate on their nature. Perhaps these cytoplasmic granules correspond to the 40 S particulate fraction (cellular ribonucleic acid of Schachman et al.[12] At a magnification of 89,000 diameters, we could find no structures comparable to the chromatophores of Vatter,[14] the site of glycogen granules of Cedergren,[3] the vesicles (fat vacuoles) of Smith,[10] or the mitochondria equivalents of Niklowitz.[20]

At D (Fig. 1) is an electron-lucid area. This we interpret as a region equivalent to a nucleus. In no instance has a nuclear membrane been seen. Vibrio fetus evidently has what Ehret [6] refers to as a one-envelope level of organization. In contradistinction to an elongate nuclear region of various bacteria as described by Caro et al.,[2] this region of V. fetus is spherical and circumscribed. The contents of the nuclear equivalent region of Vibrio fetus is finely granular, never fibrillar as in the desoxyribose nucleic acid-containing plasms reported by Kellenberger et al.[8]

Summary

The cyto-architecture of Vibrio fetus was found to be relatively simple, with a one-envelope level of organization. The cell consists of a very delicate, single-layer cell membrane; a finely granular cytoplasm with electron-dense bodies, probably poly-

Received for publication Feb. 2, 1961.

From the Departments of Zoology (Werner), and Veterinary Science (Levy), Louisiana State University, Baton Rouge, and the Electron Microscopic Laboratory of the Department of Pathology, Louisiana State University School of Medicine (Spurlock), New Orleans.

This investigation was supported by Grant PH 2549 through the Department of Pathology, Louisiana State School of Medicine, New Orleans, and the Graduate Research Council, Louisiana State University.

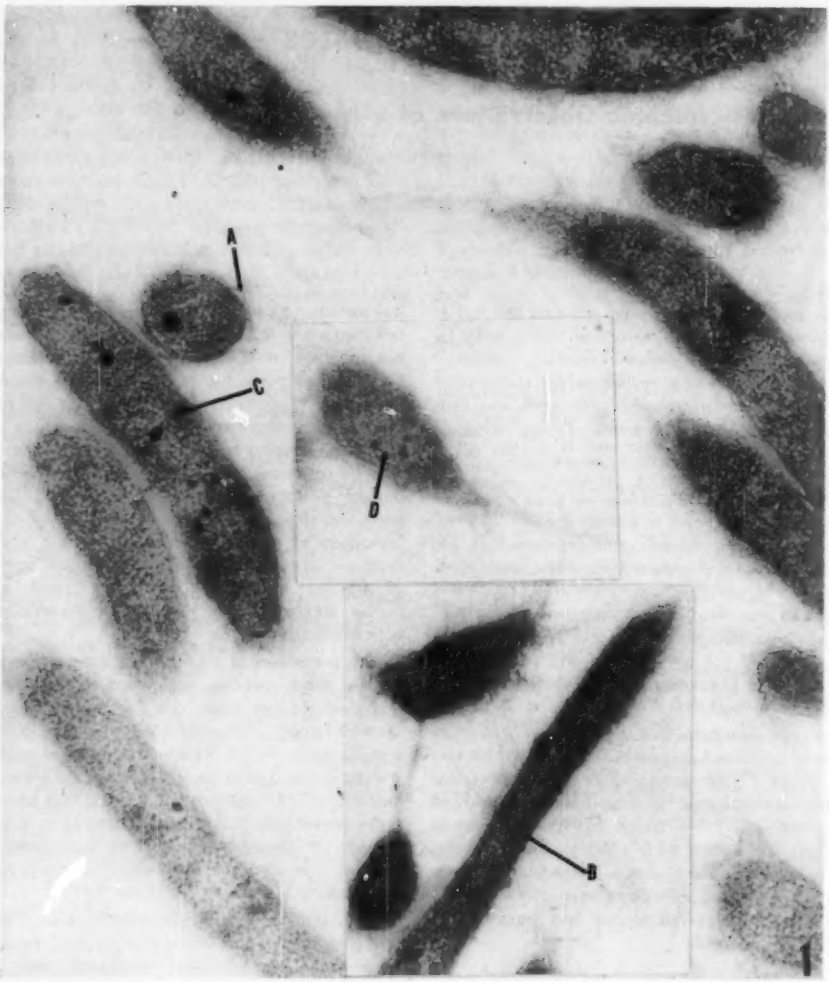


Fig. 1—Electron micrographs of ultrathin sections of Vibrio fetus. (A) Capsular residue: reacted positively to a mucoid stain; (B) cell membrane: a delicate single-layered membrane; (C) granules: presumably containing polyphosphates; (D) nuclear area: no nuclear membrane could be demonstrated.

phosphates; and an electron-lucid area, corresponding to a nucleus, devoid of a nuclear membrane.

References

[1] Caro, Lucien G., van Tubergen, R. P., and Forro, Frederick, Jr.: The Localization of Desoxyribonucleic Acid in Escherichia coli. J. Biophy. & Biochem. Cytol., 4, (1958): 491.

[2] Cedergren, B., and Holme, T.: On the Glycogen in Escherichia coli B. Electron Microscopy of Ultra-thin Sections of Cells. J. Ultra Res., 3, (1959): 70.

[3] Chapman, George B.: Electron Microscopy of Ultra-thin Sections of Bacteria. J. Bact., 78, (1959): 96.

[4] Conn, H. J., Bartholomew, J. W., and Jennison, M. W.: Staining Methods. Manual of Microbiologic Methods. McGraw-Hill Book Co., New York (1951): 29.

[5] Ehret, Charles F.: Organelle Systems and Biological Organization. Science, 132, (1960): 115.

[6] Grula, E. A., and Hartsell, S. E.: Intracellular Structures in Caulabacter Vibriodes. J. Bact., 68, (1954): 498.

[7] Hickman, Donald D., and Frenkel, Albert W.: The Structure of Rhodospirillum rubrum. J. Biophy. & Biochem. Cytol., 6, (1959): 277.

[9] Kellenberger, E., and Ryter, A.: Cell Wall and Cytoplasmic Membrane of Escherichia coli. J. Biophysic. & Biochem. Cytol. 4, (1958): 323.
[9] Kellenberger, Edouard, Ryter, Antionette, and Sechaud, Jamine: Electron Microscope Study of DNA-Containing Plasms. J. Biophysic. & Biochem. Cytol. 4, (1958): 671.
[10] Niklowitz, Werner: Mitochondrienäquivalente Bei Escherichia coli. Zentralbl Bakt. 12, (1958): 178.
[11] Rhoades, Harry E.: The Illustration of the Morphology of Vibrio fetus by Electron Microscopy. Am. J. Vet. Res. 15, (1954): 630.
[12] Schachman, H. K., Pardee, A. B., and Stanier, R. Y.: Studies on the Macromolecular Organization of Microbial Cells. Arch. Biochem. Biophysics. 38, (1952): 245.
[13] Smith, Kenneth R., and Smith, Gerald R.: An Electron-Microscope Study of Methionine Deficient Escherichia coli. J. Ultra Res. 4, (1960): 213.
[14] Vatter, A. E., and Wolfe, R. S.: Structure of Photosynthetic Bacteria. J. Bact. 75, (1958): 480.
[15] Watson, M. L.: Staining of Tissue Sections for Electron Microscopy with Heavy Metals. J. Biophys. & Biochem. Cytol. 4, (1958): 475.

SUMMARIO IN INTERLINGUA

Observationes per Microscopia Electronic in un Racia Mucoide *Vibrio fetus*

Esseva trovate que le cyto-architectura de Vibrio fetus es relativemente simple, con un nivello de organisation characterisate per un sol inveloppe. Le cellula consiste de un delicatissime e unistratate membrana cellular, de un finmente granular cytoplasma con corpores electrono-opac (probabilemente polyphosphatos), e un area electrono-lucide que corresponde a un nucleo sec que es disproviste de un membrana nucleari.

The Intestinal Fluke (Fasciolopsis buski) in a Monkey

H. A. Hartman, D.V.M., M.Sc.

The use of the rhesus monkey (Macaca mulatta) in countless medical research projects has confronted investigators with numerous parasitic species with which they must become familiar to evaluate data obtained through experimental procedures. It is the purpose of this report to describe the occurrence of the intestinal fluke Fasciolopsis buski * in the stomach of a rhesus monkey and to mention other parasites found in this monkey.

A review of medical and biological literature reveals only a single mention of F. buski ova in the monkey.[2] This report described the presence of the ova in the feces of a monkey in a group of 149 being conditioned prior to distribution to research laboratories; no mention of the adult form was made. It is possible that these were ova of F. hepatica, since they are difficult to differentiate from those of F. buski.[4] At the time of identification, it was stated that no previous report of this trematode in the rhesus monkey had been made.[1]

While it is not unusual to encounter the other species described in this paper, it was felt that the parasites observed in this single animal formed an interesting collection and were worthy of mention.

Received for publication April 28, 1961.

First Lieutenant Hartman VC, U.S. Army, is with the U.S. Army Medical Unit, Fort Detrick, Frederick, Md.

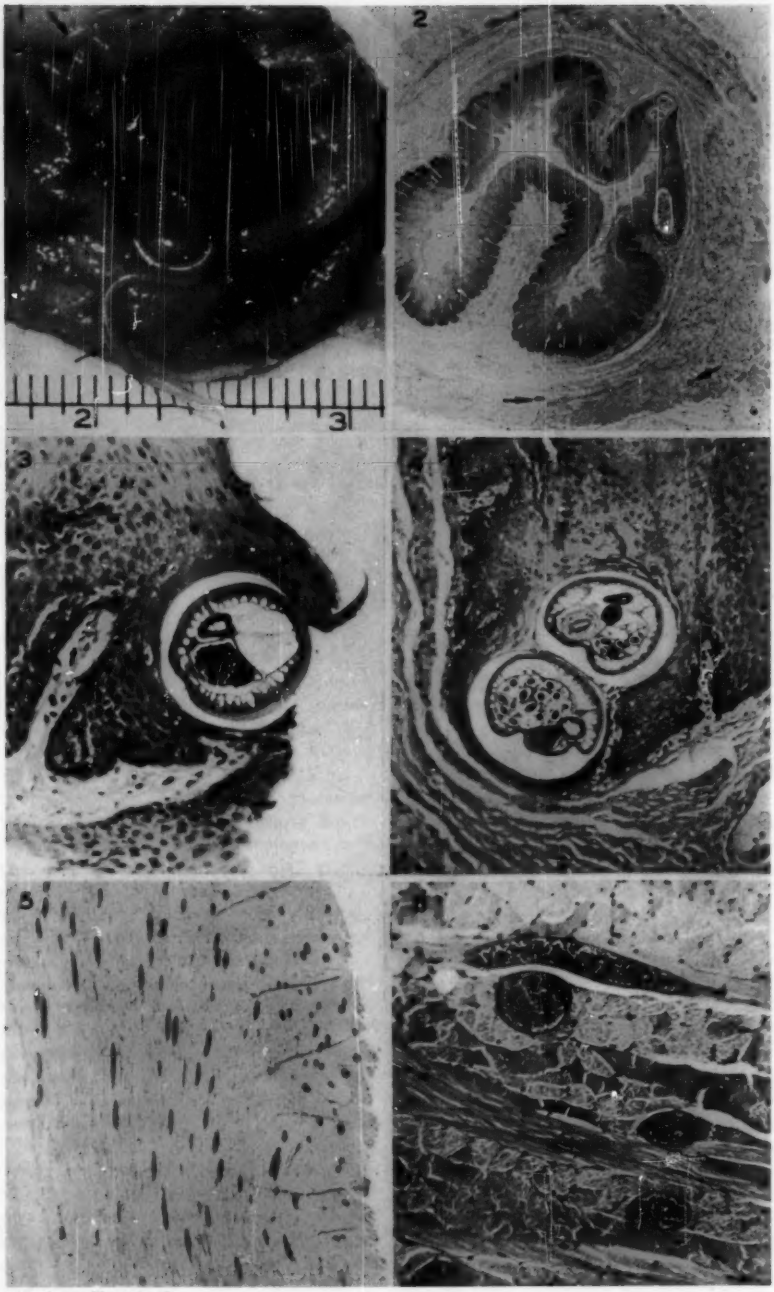
* The author thanks Dr. Chitwood and Dr. McIntosh at the Beltsville Parasitological Laboratory, USDA, for the identification of this parasite. The parasite has been catalogued in the U.S.N.M. Helminthology Collection as specimen No. 56694.

The monkey host of the parasitic species described in this report was a female M. mulatta weighing 2,500 Gm. At the time of necropsy, the monkey was in relatively good physical condition. She had been in captivity at least eight to 12 months.

Upon opening the stomach, a large blood-red trematode and 3 gray-yellow nematodes were observed attached to the gastric mucosa (Fig. 1). The trematode was ovoid and measured approximately 2 cm. at its widest point, 3 to 3 cm. long, and 3 to 6 mm. thick. It was alive and contracted vigorously while being examined and manipulated for photographic purposes. Only when immersed in fixative did the parasite release its attachment to the gastric mucosa. A few isolated focal areas of congestion, 1 to 2 mm. in diameter, were observed in the gastric mucosa. It is likely that these were former sites of attachment by the trematode since the nematodes, Physaloptera tumefaciens,[1] were deeply embedded in the mucosa. The nematodes were 4 to 5 cm. long and 2 mm. in diameter. No clinical or gross evidence of gastroenteritis attributable to these parasites was found.

Other parasites, or lesions resulting from them, observed at necropsy were Oesophagostomum sp. in the cecum and large intestine and Pneumonyssus sp. in the lung. Trematode ova were not observed in the feces.

Portions of all body tissues were collected from this monkey and placed in buffered formalin im-



U.S. Army Photograph

mediately after death for histopathologic study. They were embedded in paraffin; cut at 6 μ, and stained with hematoxylin and eosin.

During the routine histopathologic examination, other parasite species were found. Miescher's tubes of the Sarcocystis sp. were present in unusually high concentrations in muscles of the tongue, esophagus, limbs, and sublumbar region (Fig. 2, 5, 6); there were no host reactions to them. The average size of the Miescher's tubes was 80 to 90 μ in diameter. The length averaged 300 to 500 μ, occasionally reaching 1,000 to 1,100 μ. Close examination of cardiac and smooth muscle revealed no evidence of Sarcocystis infection.

Gongylonema sp.[5] was observed microscopically in the epithelia of the tongue and esophagus (Fig. 2–4). The nematodes were approximately 170 μ in diameter. No evidence of host reaction was present at either location.

Numerous granulomas resulting from the migrating larvae of the Oesophagostomum sp. were present in sections of the large intestine, cecum, and mesenteric lymph nodes. Granulomatous reactions to these parasites were found in the submucosa, muscle layers, and in well-circumscribed lesions located in the subserosa and mesentery. In several instances, wide tracts of granulomatous inflammation extended through the entire muscle wall.

Lesions of pulmonary ascariasis were found during gross and microscopic examinations. Lesions of this type are common to M. mulattā. They were characterized by bronchiolar dilatation, peribronchiolar lymphoid hyperplasia, pigment-laden macrophages, and peribronchiolar muscular hypertrophy. Parasites are frequently seen within these lesions or embedded in the thickened bronchiolar wall; however, in this animal they were present only in one unaffected bronchiole.

Examination of the stomach revealed occasional small focal ulcerations of the mucosa with small granulomatous reactions in the submucosa, probably caused by Physaloptera sp. Eosinophils were not conspicuous in adjacent tissues.

Life Cycle of Fasciolopsis buski

Fasciolopsis buski is a trematode frequently reported in man and swine in southern China, India, Sumatra, Straits Settlements, Borneo, and Thailand. It is commonly referred to as the Oriental giant intestinal fluke, or giant intestinal fluke of man and swine.[3, 4]

The adult inhabits the small intestine and occasionally the stomach. Egg production capacity is estimated at about 25,000 per day. Ova found in the stool are unembryonated and oval; they measure 130 to 140 μ by 80 to 85 μ. They have a thin transparent shell and a slightly convex operculum. Approximately three to seven weeks are required for the eggs to hatch in warm water. The infective larvae then enter snails, where they develop into sporocysts. Two generations of rediae develop from these sporocysts. The second generation produces a large number of cercariae which escape from the snail and eventually become encysted on aquatic vegetation as metacercariae.[3] The period spent in the snail is about two months.

Human infection results via the oral route during the ingestion of the water buffalo nut or water chestnut. In the Orient, the hulls of these nuts are frequently removed with the teeth. The metacercariae are swallowed and eventually develop into larvae which attach to the mucosa of the small intestine and develop into mature trematodes in about three months.[3] The use of human excreta as fertilizer for aquatic plants maintains a high level of infective larvae for the molluscan stage of the life cycle.[4]

Because of the obscure history of the average rhesus monkey from the time of capture to arrival at the laboratory, one can only speculate as to the time and source of infection of the monkey reported here.

Legend for Figures on Opposite Page

Fig. 1—Fasciolopsis buski and Physaloptera tumefaciens attached to the gastric mucosa of the monkey. The scale is in inches.

Fig. 2—Gongylonema sp. in the esophagus; cross sections of 3 nematodes are visible in the epithelium. Miescher's tubes of Sarcocystis are present in peripheral skeletal muscle (arrows). H & E stain; x 12.

Fig. 3—Gongylonema sp. in tongue epithelium. H & E stain; x 370.

Fig. 4—Gongylonema sp. in esophageal epithelium. H & E stain; x 70.

Fig. 5—Sarcocystis sp. in skeletal muscle; notice high concentration of Miescher's tubes. H & E stain; x 12.

Fig. 6—Sarcocystis sp. in skeletal muscle. H & E stain; x 465.

Pathogenicity in Man

Small numbers of F. buski produce few or no intestinal signs; however, infections are frequently caused by dozens or even hundreds of adults. The presence of large numbers of this trematode produces mucosal ulceration, acute obstruction, and absorption of toxic metabolites. Typically, anorexia, nausea, and vomiting occur; edema of the face, abdomen, and lower extremities and extreme eosinophilia are common. Fetid diarrhea, containing partially digested food, also occurs.[3, 4]

Summary

The intestinal fluke, Fasciolopsis buski, was found in the stomach of a rhesus monkey. It is also a parasite in man. Other parasitic species observed in this monkey were Physaloptera, Sarcocystis, Gongylonema, Oesophagostomum, and Pneumonyssus.

References

[1] Chitwood, M. B.; Beltsville, Md: Personal communication, October, 1960.

[2] Dieterich, W. H.: The Physical Examination of Rhesus Monkeys. J.A.V.M.A. 127, (August, 1955): 137–141.

[3] Faust, E. C.: Animal Agents and Vectors of Human Disease. Lea & Febiger, Philadelphia (1956): 369.

[4] Manson-Bahr, P. H.: Manson's Tropical Diseases. Williams & Wilkins Co., Baltimore (1956): 937.

[5] Ruch, T. C.: Diseases of Laboratory Primates. W.B. Saunders & Co., Philadelphia (1959): 175.

SUMMARIO IN INTERLINGUA

Le Distoma Intestinal *(Fasciolopsis buski)* in un Simiano

Le distoma intestinal, Fasciolopsis buski, esseva trovate in le stomacho de un rheso. Iste distoma es etiam un parasito in humanos. Altere species parasitic observate in iste simiano esseva Physaloptera, Sarcocystis, Gongylonema, Oesophagostomum, e Pneumonyssus.

The Protozoan Parasite, Klossiella equi, in the Mexican Burro

H. A. Hartman, D.V.M., M.Sc.

The intracellular phases in the life cycle of the protozoan parasite, Klossiella equi, have been described in detail in the zebra[2] and American jackass.[5] Other members of the same genus have been reported and described in the mouse, guinea pig, and jerboa.[2, 6, 7] It is the purpose of this report to describe K. equi in burros, to compare the observations made in the burro with those previously described in the zebra and American jackass, and to illustrate sporozoites within the mature spores.

The burros were part of a herd maintained for laboratory purposes. As nearly as can be determined, these animals originated in the states of Tamaulipas and Nueva Leon, Mexico. The animals had been in the United States for two to 18 months.

Following experimental use, postmortem examinations were performed immediately after death. Tissues were placed in buffered formalin. Sections were cut 6 μ thick and stained with hematoxylin and eosin.

Routine histopathologic examinations were performed on 14 animals, 13 of which were males. Klossiella equi in all stages of intracellular development was recognized in 8 male animals. Random sections had been taken from both kidneys. Sections of liver and spleen were carefully studied; results were negative for this parasite.

Received for publication April 28, 1961.

First Lieutenant Hartman, VC, U.S. Army, is with the U. S. Army Medical Unit, Fort Detrick, Frederick, Md.

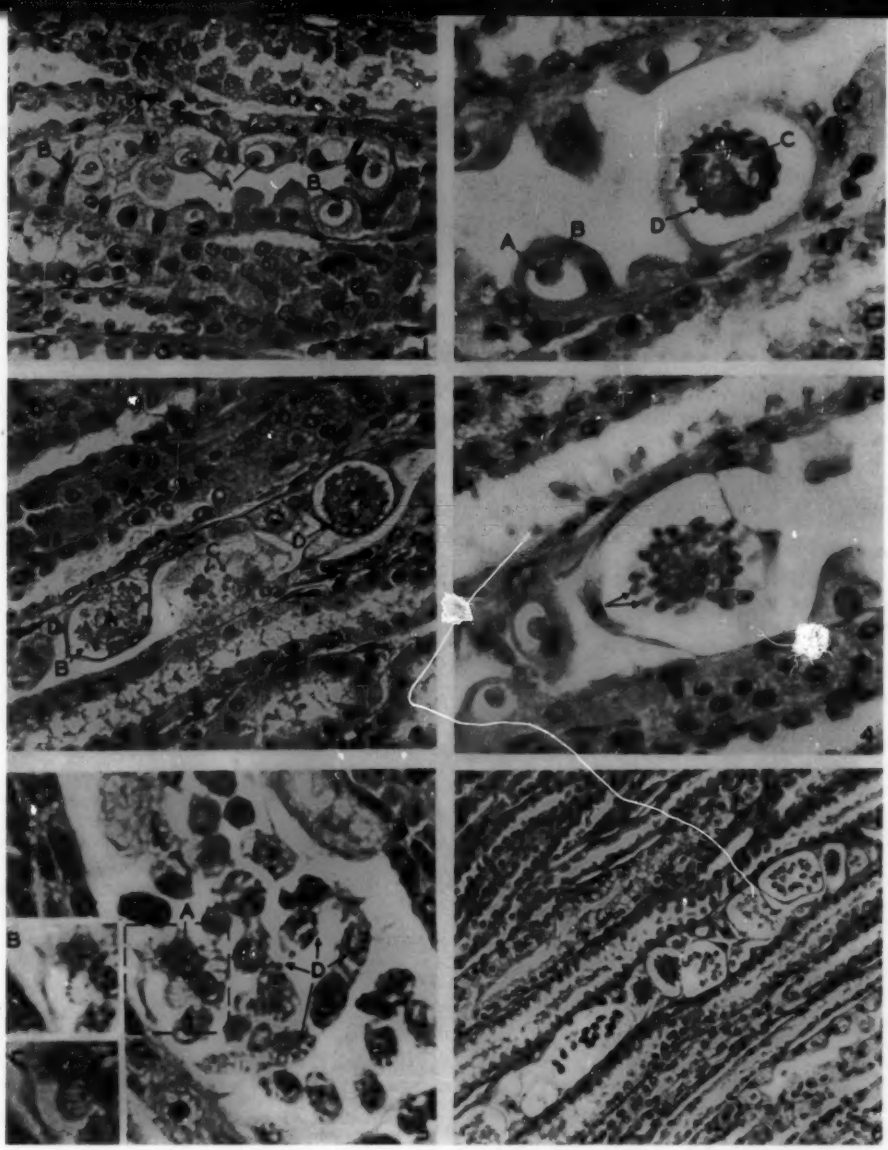
Intracellular Stages in the Development of Klossiella equi

A summary of the intracellular (sporogony) stages previously described[2, 5] and illustrated in this report by the author, follows:

Uninuclear Phase.—A mass of reticulated protoplasm about 7 by 9 μ is present in a cytoplasmic vacuole of an enlarged renal epithelial cell (Fig. 1A). Nuclei are observed in the periphery of these masses (Fig. 2B). In approximately 25% of the vacuoles, 1 μ signet-ring bodies are adjacent to the reticulated masses (Fig. 1B). It was suggested that these were the micro and macrogametocytes.

Uninuclear Englargement Phase.—The protoplasmic masses enlarge and chromatin material becomes more conspicuous (Fig. 2C); it gradually assumes a peripheral arrangement.

Peripheral Budding Phase.—As the intracytoplasmic masses increase in diameter, bud-like projec-



U.S. Army Photograph

Fig. 1—Several early uninuclear masses within vacuolated epithelial cells (A). Minute basophilic (signet-ring) bodies adjacent to protoplasmic masses (B). H & E stain; x 410.

Fig. 2—Peripheral nuclear mass (A) in early uninuclear phase (B). Larger spherical mass of reticulated protoplasm (C) with peripheral accumulations of chromatin (D). H & E stain; x 730.

Fig. 3—Early budding stages—large central protoplasmic masses (A) with small peripheral buds (B); immature spores from a ruptured cell (C); notice greatly distended cell wall (D). H & E stain; x 425.

Fig. 4—Intermediate budding stage—peripheral arrangement of buds (A), radiating from a central protoplasmic mass (B). Each bud contains dark nuclear mass. H & E stain; x 585.

Fig. 5—Mature spores containing neatly arranged sporozoites (A). The two inserts (B & C) are focused at different levels in the same field as (A) to illustrate better the individual sporozoites and nuclear masses in each. Irregular arrangement of sporozoites nuclei in other spores illustrated (D). H & E stain; x 830.

Fig. 6—Renal tubule displaying all stages of intracellular development. H & E stain; x 190.

tions develop at the periphery (Fig. 3B and 4A). Within each bud there is a deeply basophilic mass of nuclear material. The buds increase in size and develop into spores. As the spores develop, the central mass of protoplasm gradually diminishes.

Oocyst Phase.—The host epithelial cell appears as a large sac with numerous spores (25 to 40) (Fig. 5, 6).

Sporozoite Phase.—Within each spore, the discrete basophilic granules (Fig. 5) are thought to be sporozoites, although they are stained with difficulty.

The forms of K. equi present in the renal epithelium of the loop of Henle and medullary rays are thought to be the sporogony phase.

Discussion

The K. equi observed in the burros reported here were identical to those described in the zebra and American jackass.

The signet-ring bodies previously described [2] in 25% of the vacuolated epithelial cells were found in several animals (Fig. 1B). However, the frequency of their occurrence was irregular. In some burros, the bodies were difficult to find, in others they were conspicuous; in some instances two or three bodies were observed per vacuole. An accurate estimate of the number of vacuoles containing the minute bodies could not be made.

Numerous cysts containing spores were observed, but relatively few neatly arranged clusters of sporozoites were seen within individual spores (Fig. 5A, B, C). In most cases, the spores contained numerous, discrete basophilic granules which appeared to be the nuclei of the sporozoites (Fig. 5D). The fact that only a few cysts displayed this neat arrangement of sporozoites could be the result of several factors, including the presence of more numerous immature spores or early cyst rupture.

It has been suggested that the debris present in the collecting tubules contains mature sporozoites.[3] The amorphous debris observed in the tubules of Klossiella-parasitized and nonaffected burros, was similar in nature and amount. If one assumes that the parasitized renal cell ruptures to release the intact spores or the mature sporozoites into the lumen, the existing tubular debris could mask the sporozoites. No forms comparable to spores or sporozoites were observed in the lumens.

The following special stains were used in an attempt to detect forms in the tubules and to study the intracellular stages: periodic acid Schiff, Gridley fungus, Brown and Brenn, Giemsa, oil red O, Gomori's methanamine silver, and the Ziehl-Neelsen stain for acid-fast bacilli. Only the oil red O was specific for the intracytoplasmic stages; however, no evidence of parasitic forms was found in the lumens. In general, the hematoxylin and eosin stain proved superior for the detection and morphologic examination of this parasite.

Although there was considerable difference in the degree of infection among the 8 animals, wherever the parasite was observed, the entire sporogenic cycle, as previously summarized, could be seen. In some animals, sites of parasitism were difficult to detect, while in others they were conspicuous and widespread (Fig. 6).

Although it is obvious that infection by K. equi results in destruction of renal epithelium, there is no evidence of resulting inflammatory response. There was no significant dilatation of tubular structures proximal to the site of parasitic lesions, which in many cases appeared capable of occluding the lumen (Fig. 3, 6).

Summary

Klossiella equi, the protozoan parasite previously described in the renal epithelium of the zebra and the American jackass, was observed in the kidneys of 8 of 14 Mexican burros. The stages of sporogony observed in the kidneys were comparable to those described in the other members of the equine species.

References

[1] Kudo, R. R.: Protozoology. 3rd ed. Charles C Thomas, Springfield, Ill., 1946.

[2] Newberne, J. W., Robinson, V. B., and Bowers, N. E.: Histological Aspects of K. equi in the Kidney of a Zebra. Am. J. Vet. Res., 19, (April, 1958): 304–307.

[3] Pearce, L.: Klossiella Infection of the Guinea Pig. J. Exptl. Med., 23, (1916): 431–442.

[4] Seidelin, H.: Klossiella Species in the Kidney of a Guinea Pig. Ann. Trop. Med. & Parasitol., 8, (1914): 553–564.

[5] Seibold, H. R., and Thorson, R. E.: Klossiella equi N. Sp. (Protozoa: Klossiellidae) from the Kidney of an American Jack. J. Parasitol., 41, (1955): 285–288.

[6] Smith, T., and Johnson, H. P.: On a Coccidium Parasitic in the Renal Epithelium of the Mouse. J. Exptl. Med., 6, (1902): 303–316.

[7] Wenyon, C. M.: Protozoology; A Manual for Medical Men, Veterinarians, and Zoologists. Baillière, Tyndall & Cox, London, 1926.

SUMMARIO IN INTERLINGUA

Le Parasito Protozoic *Klossiella equi* in le Burro Mexican

Klossiella equi, le parasito protozoic previemente describite como occurrente in le epithelio renal del zebra e del asino american, esseva observate in le renes de 14 burros mexican. Le stadios de sporogonia observate in le renes del burros esseva comparabile a illos describite pro le altere membros del specie equin.

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave, Chicago 5, Illinois.

Disposable Plastic Aprons

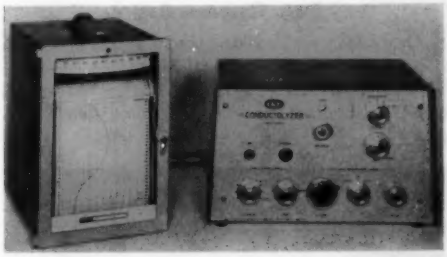
A long, disposable plastic apron (27 by 42 inches) is available from Busse Plastics Co. The apron, of

opaque white vinyl, has bib and waist ties, has a linen-like embossed surface to retard the flow of drippings, and resists water chemicals, soiling and staining.

Conductolyzer

A new instrument for automatic resistance measurements of streaming solutions has been designed by LKB Instruments, Inc. The 5300 Conductolyzer Conductivity Analyzer can be used where solution composition causes electrical conductance changes, such as from chromatography and electrophoresis columns.

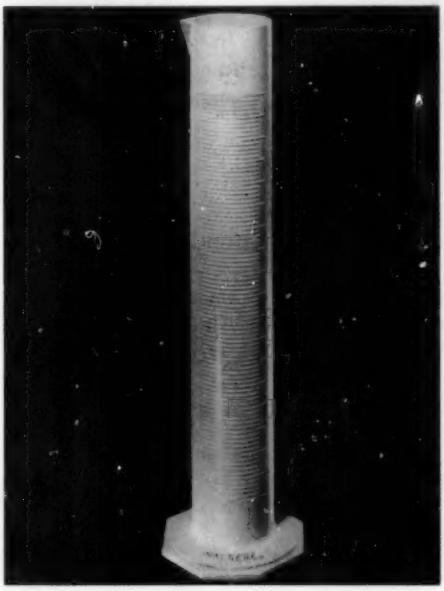
Three continuous-flow cells cover a range of 5×10^{-6} to 4 mhos, and have small hold-up volumes of from 0.02 to 0.05 ml. A discriminator in the circuit reduces the influence of varying cell



resistance. If impedance measurements are required, connection can be made to a Z jack and qualitative measurements can be gathered by simultaneous comparison of R and Z curves.

Polypropylene Cylinders

A redesigned series of graduated cylinders, manufactured by Nalgene Co., Inc., are made of virgin polypropylene. The cylinders are accurate, break-



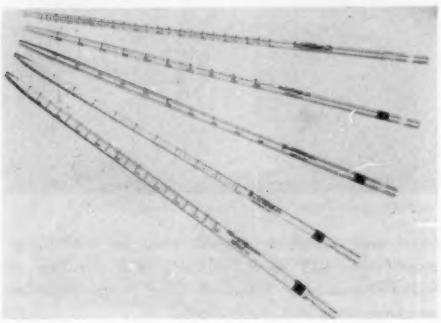
proof, and chemical-resistant; they are available in seven sizes (25 to 2,000 ml.) and conform to National Bureau of Standards Class A specifications for dimensions and graduations.

Dog Carcass Bag

An inexpensive bag for disposal of dog carcasses is produced by Busse Hospital Products. The bag, 30 by 48 inches, is made of heavy, laminated, creped, waterproof paper with leakproof seams.

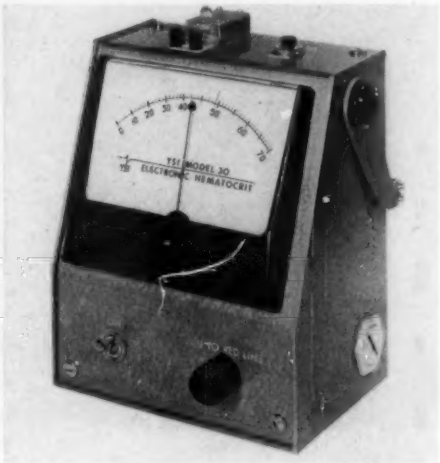
Color-Coded Pipets

Color-coding of pipets for size identification has been announced by Kimble Glass Co. Thus far, 2 pipets, a plain serologic pipet and a serologic pipet for cotton plugging, are available in color. Colored bands, fused to the glass, allow immediate identification of pipet size, accuracy in size selection, and simplified sorting.



Transistorized Micro-Hematocrit

An instantaneous, direct-reading, transistorized micro-hematocrit, the YSI Model 30 Electronic Micro-Hematocrit, has been released by the Yellow Springs Instrument Company. The unit, which is battery-powered and portable, is based on the insulating capacity of red blood cells. Sufficient blood, whole or heparinized, is pipetted into the hematocrit cell to fill the area between the electrodes (0.02 cc.). The cell is then placed between brass contacts, and a direct reading (in hematocrit per cent units) is immediately available.



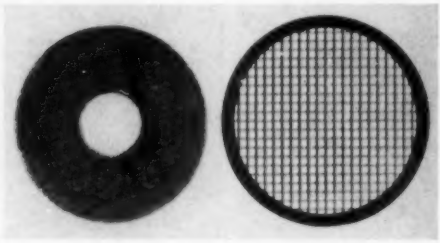
Warming Pad for Baby Pigs

A thermostat-controlled warming pad (Pamper Pad, Inc.) maintains ideal body temperatures for baby pigs by supplying heat in balance with the heat generated by the baby pig. The mat is made of a rubber compound reinforced with nylon and rayon. A bitter, non-toxic substance is milled into the rubber to discourage chewing by the sow or pigs. The mat and the steel-armored plug cable are re-

sistant to water, acid, and oil; the mat is immersible and nonporous to facilitate cleaning and sterilization.

Mounting Grids for Ultrathin Sectioning

Grids for mounting ultrathin grids for electron microscopy are available from LKB Instruments,



Inc. The grids, made of copper by an electrolytic process, have a solid rim for easy handling. In addition to standard mesh sizes from 75 to 400, special patterns are available, such as grids with single circular openings for serial mountings.

Mobile Apparatus Support

A Mobile-Rack, manufactured by Labline, Inc., is used for supporting heavy laboratory apparatus assemblies such as distillation columns and extractors. Heavy double-sided channels and support

rods are bolted together with slip-proof clamps to form a rigid frame which can be adjusted quickly. The unit can be used from both sides simultaneously and is mounted on four rubber-tired casters with built-in brakes which can be locked to maintain the rack position.

Improved Laboratory Beakers

Made of light-weight, laboratory-grade polypropylene, beakers manufactured by the Nalge Company now incorporate an oval sandblasted area for easier identification marking. In addition, a new 2,000-ml. size has been added to the line, increasing the size range from 30 to 2,000 ml.

Bind Your Copies

of the

JOURNAL OF THE AMERICAN VETERINARY MEDICAL ASSOCIATION

and

AMERICAN JOURNAL OF VETERINARY RESEARCH

The publishers have effected an arrangement with one of America's foremost bookbinders by which subscribers can have their journals economically bound, to the publisher's specifications.

Your copies of the JOURNAL of the American Veterinary Medical Association will be bound in the finest grade black washable buckram, with your name imprinted on the front cover. Price $4.95 per volume.

Ship journals parcel post. Bound volumes will be returned transportation prepaid. Full remittance must accompany order.

Publisher's Authorized Binding Service, Ltd.

Binders of all scientific and technical journals.

430 W. Erie St., Chicago 10, Ill.

AMERICAN JOURNAL OF VETERINARY RESEARCH

★

Volume XXII
NUMBERS 86, 87, 88, 89, 90, 91
JANUARY-DECEMBER, 1961

★

CONTENTS COPYRIGHTED 1961 BY THE AMERICAN VETERINARY MEDICAL ASSOCIATION

Published by the

AMERICAN VETERINARY MEDICAL ASSOCIATION

600 S. Michigan Ave., Chicago, Ill.

Index of Authors

Aanes, W. A., 47.
Abdalla, A., 1012.
Abinanti, F. R., 13.
Adams, W. V., 303.
Adcock, Jerry L., 655.
Adler, H. E., 382.
Alicata, Joseph E., 704.
Allen, Robert C., 558, 564, 995.
Amerault, T. E., 564.
Andrews, F. N., 462.
Andrews, M. F., 1026.

Baker, Norman F., 590.
Baldwin, D. E., 224.
Barakat, M. Z., 1012.
Baron, R. R., 899.
Beasley, J. N., 85.
Belding, Ralph C., 271.
Bell, D. S., 686, 1015.
Bello, Thomas R., 597.
Berger, L. R., 1000.
Bharadwaj, Madan B., 764.
Bishop, J. A., 1000.
Bittle, J. L., 374.
Blankenship, Lytle, 156.
Blenden, D. C., 944, 1081.
Blincoe, Clifton, 422.
Blommer, Earl, 388.
Bogdon, Thomas R., 757.
Botts, R. P., 355.
Brisson, G. J., 990.
Burns, M. J., 147.
Bussell, Robert H., 1069.
Byrne, Robert J., 266.

Calhoun, M. Lois, 764.
Callantine, M. R., 462.
Callstrom, R. C., 72.
Campbell, Berry, 37.
Campbell, L. A., 587.
Carbrey, E. A., 573.
Carter, Paul B., 753.
Chang, P. W., 93.
Christensen, John F., 590.
Chute, H. L., 99.
Clark, C. H., 147, 370.
Clark, Lawrence G., 949.
Cohen, Daniel, 637.
Cole, Clarence R., 53, 473, 482, 663.
Conner, G. H., 487.
Copaira, Marcos, 673.
Cornelius, C. E., 683, 1000.
Cottral, G. E., 224.
Cox, B. F., 224.
Cuba-Caparo, Alberto, 673.

Dacres, W. G., 570.
Dardiri, A. H., 93.
Davis, Charles L., 932.
Davis, J. W., 736.
Dean, Donald J., 644.
De la Vega, Elmo, 673.
DeLay, P. D., 533.
Detweiler, D. K., 355.
Dimopoullos, George T., 986.
Doll, E. R., 731.
Dorfman, Ralph I., 313.
Dow, C., 352.
Drehle, Loyd E., 921.
Drudge, J. H., 128, 1106.
Drury, A. R., 975.
Dunne, H. W., 391.
Dziuk, H. E., 287.

Eisa, Amin M., 708.
Elam, G. W., 128, 1106.
Ellis, Edwin M., 610.
Eisner, Yoko Yamakawa, 753.
Emery, J. B., 374.
Englehard, Warren E., 396.
Eveleth, D. F., 1026.
Ewing, S. A., 606, 1077.

Fassel, Hester, 117.
Feaster, J. P., 887.
Fellowes, O. N., 779.
Firehammer, B. D., 449.
Fisher, E. W., 279.
Fleming, L. W., 970.
Foote, Lon E., 877.
Fox, Lauretta E., 123.
Frank, F. W., 899.
Frank, Norma A., 32.
Fries, G. F., 487.
Fry, D. E., 93.
Funnell, H. S., 80.

Gaafar, S. M., 746.
Gabel, Albert A., 720.
Gainer, J. H., 882.
Galton, M. M., 650, 1085.
Gates, D. W., 982.
Geer, Jack C., 877.
Geil, Robert G., 932.
Geleta, J. N., 713.
Gennaro, J. F., 887.
Gillespie, James H., 1069.
Gilmore, Viriginia, 23.
Goldberg, Herbert S., 1081.
Good, A. L., 156, 1093.
Goode, E. R., Jr., 564.
Gordon, Norma, 142.
Goulet, Normand R., 1112.
Greene, J. W., 811.
Gregory, P. W., 693.
Griesemer, Richard A., 473, 482.
Griner, L. A., 429, 443, 447.
Gustafson, D. P., 741.

Hammon, William McD., 637.
Hanson, Lyle E., 274, 580.
Hare, W. C. D., 825.
Hartman, H. A., 1123, 1126.
Hawk, H. W., 689, 1117.
Hawkins, W. W., Jr., 412, 416, 419.
Heuschele, W. P., 683.
Higinbotham, W. A., 887.
Hirata, Arthur A., 158.
Hoag, Warren G., 150.
Hoerlein, Alvin B., 470.
Hoerlein, B. F., 1041.
Holbrook, A. A., 713.
Hollister, Charles J., 949.
Howes, J. R., 887.
Hubben, K., 355.
Hupp, E. W., 406.

Ishizaki, Ryotaro, 250.

James, Garth A., 388.
Jarrett, W. F. H., 186, 352, 492.
Jennings, F. W., 186, 352, 492.
Jensen, Marcus L., 388.
Jensen, Rue, 43, 169, 601.
Jha, S. K., 912, 915, 948.
Johnson, Catherine B., 731.
Johnson, R. Bernal, 330.
Johnston, R. F., 912, 915, 948.
Jones, L. Meyer, 544, 553.
Julian, L. M., 693.
Jungherr, Erwin L., 937.
Juszkiewicz, Teodor, 537, 544, 549, 553.

Kaneko, J. J., 683.
Karstad, Lars, 227.
Karzon, David T., 1069.
Keeler, Richard F., 617.
Keith, T. B., 899.
Kemeny, Lorant, 921.
Kempe, C. Henry, 852.
Kitchell, R. L., 37, 72.
Kling, J. M., 370.
Knapp, Fred W., 332.
Kobayashi, Shigeo, 1066.
Koestner, Adalbert, 53.
Koths, M. E., 864, 867.
Kreier, Julius P., 790, 795.
Kresse, Joseph I., 949.
Kronfeld, D. S., 496, 587.
Kume, Seiji, 1066.
Kuppuswamy, P., 1081.
Kuttler, Kenneth L., 422.

Lagacé, André, 686, 1015.
Lambert, G., 564.
Landram, James F., 893.
Langille, James, 1057.
LaPlaca, Michele, 852.
Larsen, Aubrey B., 204, 1074.
Larson, L. L., 37.
Layton, J. M., 104.
L'Ecuyer, C., 67, 1020.
Leland, S. E., Jr., 128, 1106.
Levy, Helen, 1121.
Linder, Donna, 308.
Loan, R. W., 741.
Lobl, K., 217.
Lovelace, S. A., 449, 617.
Luedke, A. J., 391.
Luginbuhl, Hansruedi, 1030.
Lumb, W. V., 912, 915, 948, 1097.
Lynch, John E., 324.
Lynd, Frederick T., 704.
Lyons, E. T., 1106.

Maisel, John, 852.
Mansfield, Manford E., 470.
Manthei, C. A., 564.
Marble, Donald W., 422.
Marshak, Robert R., 949.
Marty, Edward W., Jr., 770.
Mason, Marcus M., 1057.
May, Hilda J., 926.
McCollum, William H., 731.
McDaniel, L. S., 99.
McDonald, L. E., 209.
McIlwain, P. K., 1026.
McIntyre, W. I. M., 186, 352, 492.
McMillen, J. K., 374.
Meinecke, C. F., 209.
Meinershagen, W. A., 899.
Mejia, M. J., 502, 698.
Menges, Robert W., 1085.
Merilan, C. P., 944.
Merkal, Richard S., 1074.
Miller, Victor A., 43, 169.
Millian, Stephen J., 396.
Miner, M. L., 388, 753.
Molello, J. A., 169.
Mooro, R. W., 85.
Morrison, S. M., 327.
Moscovici, Carlo, 852.
Motohashi, Tsunemasa, 236.
Mott, L. O., 982.
Moulton, W. M., 18.
Moxon, A. L., 686.
Mulligan, R. M., 345.
Mulligan, W., 186, 352, 492.
Murphree, R. L., 406.
Murty, D. Krishna, 274, 783.
Muth, O. H., 298, 466.

Nelson, Dallas L., 332.
Nichols, R. E., 117.
Nielsen, S. W., 663.
Norcross, Neil L., 775.
Norton, Horace W., 795.

Ogata, Manabu, 266
Ohishi, Isamu, 1066.
Oldfield, J. E., 466.
Oliver, W. T., 80.
Osebold, John W., 590.

Pace, H. B., 406.
Packer, R. A., 573
Padgett, George A., 749.
Palmer, Dan F., 139.
Pangborn, J., 1000.
Paton, Iain M., 906, 970.
Patterson, D. F., 355.
Patty, Richard E., 775, 926.
Pavia, Rose A., 637.
Perk, K., 217.
Peterson, Donald A., 330.
Petty, M. F., 1041.
Pier, A. C., 502, 698.
Pike, Robert M., 906.
Pilcher, K. S., 298.
Pirtle, E. C., 104
Plastridge, W. N., 864, 867.
Plumer, G. J., 13.
Pounden, W. D., 32, 686, 1015.
Prier, J. E., 614, 1112.

Rajya, B. S., 189.
Ramirez, M. A., 995.
Reber, Elwood F., 795.
Reynolds, I.M., 800.

Rigdon, R. H., 379.
Ristic, Miodrag, 109, 783, 790, 871.
Roberts, E. D., 67, 1020.
Robertstad, G. W., 327.
Roby, T. O., 982.
Rosen, W. G., 117.
Rosenfeld, George, 313.
Rosenkrantz, Harris, 1057.
Roth, Earl E., 308.
Rozemeyer, H., 533.
Rubin, Robert, 708.

Sadler, W. W., 382.
Samejima, Togo, 236.
San Clemente, C. L., 975.
Sanger, V. L., 271.
Sauer, F., 990.
Saxena, Sohan P., 470.
Schalm, O. W., 23.
Schipper, I. A., 401.
Schoenhard, D. E., 749.
Schricker, R. L., 580.
Schubert, J. R., 466.
Schulze, Mary Louise, 906.
Scott, Walter L., 877.
Scrivner, L. H., 899.
Sellers, A. F., 287.
Sesaki, Kizo, 319.
Sharp, N., 370.
Sharp, N. C. C., 186, 492.
Sharpless, George R., 937.
Shaver, Robert J., 893.
Sherman, Inez, 644.
Shimizu, Takehiko, 250.
Singh, C. M., 189.
Skold, Bernard H., 601.
Smibert, R. M., 736.
Smith, R. E., 800.
Smith, W. Whitney, 388, 753.
Solomon, John J., 975.
Sperling, F. G., 154, 813.
Spurlock, Ben O., 1121.
Stevens, C. E., 287.
Stewart, G. F., 382.
Stob, M., 462.
Stoliker, H. E., 650.
Stone, S. S., 18.
Stowe, C. M., 156, 1093.
Straub, M., 811.
Stromberg, M. W., 72.
Stubbs, E. L., 154, 813.
Swanson, L. E., 123.
Swingle, K. F., 412, 416, 419.
Switzer, W. P., 67, 1020.
Sykes, J. F., 689, 1117.

Tajima, Masanori, 236, 250.
Tessier, J., 779.
Theilen, G. H., 23.
Thomas, J. Alan, 753.
Thompson, Samuel W., 757, 932.
Todd, A. C., 606, 1077.
Trainer, D. O., 227.
Trautman, R. J., 811.
Truszczynski, Marian, 836, 839, 846.
Tubiash, Haskell S., 807.
Turner, G. D., 689, 1117.
Tyagi, R. P. S., 1097.
Tyler, W. S., 693.

Ungar, Frank, 313.

Van Dresser, W. R., 995.
Vardaman, Thomas H., 204.

Wade, A. E., 123.
Wallbank, A. M., 813.
Watkins, J. R., 85.
Watrach, A. M., 109.
Watson, D. F., 995.
Weide, K. D., 1015.
Werner, Henry James, 1121.
White, F. H., 650.
Willers, E. H., 502, 698.
Williams, Holman E., 902.
Williams, L. F., 867.
Wilson, Benjamin J., 961.
Wilson, Christina H., 961.
Wilson, James C., 731.
Winn, John F., 139.
Winter, A., 227.
Woodley, C. H., 370.
Wyant, Z. N., 128, 1106.

Yamamoto, R., 382.
Yates, V. J., 93.
Yohn, David S., 637.
York, C. J., 374.
Yost, Donald H., 757.
Young, Stuart, 412, 416, 419.

Zahran, G. E. D., 518, 527.

Index of Subjects

A

Abortion Virus, Electron Microscopy of Equine, 250.
Achondroplasia, Bovine. III. Standards of Metacarpal Indexes for Achondroplastic, Brachycephalic Dwarfs and Controls, 693.
Acridine Orange: Comparisons with the Complement Fixation Test and Wright's Stain, Demonstration of Anaplasma marginale with the Fluorescent Dye, 882.
Adenomatosis of Sheep—Metastasizing Bronchiolar Tumors, Pulmonary, 673.
Agar Gel Diffusion-Precipitation Test, The. The Antigenic Structure of Virulent and Avirulent Strains of Erysipelothrix rhusiopathiae. III., 846.
Agar Plates as an Aid for the Isolation of Leptospires, The Use of, 308.
Agglutination Reactions for Bovine Brucellosis, A Heat-Inactivation Test for Differentiating Specific and Nonspecific, 564.
Albumin in Brains of Clostridium perfringens Type D Intoxicated Lambs, Distribution of I^{131} Radioiodinated Serum, Enterotoxemia of Sheep. II, 443.
Aldolase During Migration of Larval Ascaris suum in Swine, Serum Transaminase and, 1026.
Alfalfa Samples from Bloat-Producing Pastures, Biological Assays of Extracts of, 287.
Alimentation, Use of Emulsified Fat for Intraperitoneal, 147.
ANAPLASMA MARGINALE:
in the Bovine Erythrocyte, Electron Microscopy of, 877.
Studies on the Morphology and Respiration of, 298.
with the Fluorescent Dye, Acridine Orange; Comparisons with the Complement-Fixation Test and Wright's Stain, Demonstration of, 882.
ANAPLASMOSIS:
The Comparative Susceptibility of Calves and Adult Cattle to Bovine, 982.
Erythrocyte Survival in Experimental, 590.
Studies in. II. Electron Microscopy of Anaplasma marginale in Deer, 109.
Studies in. III. An Autoantibody and Symptomatic Macrocytic Anemia, 871.
Studies in. IV. Development of the Causative Agent in Deer Erythrocytes Transfused into Calves, 790.
Ancylostomiasis in Dogs. A Quantitative Measurement of the Blood Loss Caused by, 370.
Anemia, An Autoantibody and Symptomatic Macrocytic Study in Anaplasmosis. III, 871.
ANESTHESIA:
and the Changes Taking Place in Plasma pH and Plasma CO_2 Content, Observations of the Disturbance of Respiration of Cattle, Horses, Sheep, and Dogs Caused by Halothane, 279.
Anesthetized Deer, Cardiac Output and Related Values in, 156.
in Equine Animals, Intravenous Ether, 720.
Anthelmintic Activity of an Organic Phosphate in Cattle and Sheep, 893.
Anthelmintic in 8 Yearling Hereford Calves, A Critical Evaluation of Bephenium Hydroxynaphthoate as an, 708.
Antibiotic Mediums for the Isolation of Vibrios from Bull Semen, 867.
Antibiotic Sensitivity of Bovine Vibrios, 864.
Antibiotic Technique for Identification of Leptospiral Serotypes, Fluorescein-Labeled, 570.
Arteritis Virus in Monolayer Cultures of Equine Kidney, Propagation of Equine, 731.
Ascaris Eggs in 3 Different Levitation Solutions, Comparison of the Flotation of Metastrongylus and, 597.
Ascaris suum in Swine, Serum Transaminase and Aldolase During Migration of Larval, 1026.
Ascorbic Acid in Rats During Simulated Transportation Conditions at Normal and High Temperatures, Effects of Chlorpromazine and, 544.
Ascorbic Acid in Resisting Heat Stress in Rats, Effects of Chlorpromazine, Reserpine, and, 537.
AVIAN SPECIES:
Broilers and Caponettes Vaccinated with Killed-Virus and Live-Virus Vaccines, Newcastle Disease Immunity in, 93.
Caponettes Vaccinated with Killed-Virus and Live-Virus Vaccines, Newcastle Disease Immunity in Broilers and, 93.
Chicken—A Pathologic Study, Pendulous Crop in the, 379.
Chicken Eggs, Propagation and Modification of 3 Immunologic Types of Virus in Embryonating. Foot-and-Mouth Disease Virus. II, 527.
Chicken Embryos and Chicks, Foot-and-Mouth Disease Virus—Its Behavior in Cattle After Serial Passages in, 533.
Chicken Plasma, Enzyme Activity Levels in, 99.
Chicken Sarcoma (Strain 13) by Vent Exposure, Study of, 813.
Chicken Sarcoma (Strain 13) Placed in the Bursa of Fabricius, Study of, 154.
Chickens by Catheterization, Exsanguination of, 158.

Chickens, Relationships Between Eastern and Western Equine Encephalomyelitis Viruses as Demonstrated by the Hemagglutination-Inhibition Antibody Response of Experimentally Infected, 266.
Chicks, Propagation of 3 Immunologic Types of Virus in, Foot-and-Mouth Disease Virus. I, 518.
Duck Hepatitis. A Modified Microgel Diffusion Method and Its Application in the Study of the Virus of, 274.
Newcastle Disease Immunity in Broilers and Caponettes Vaccinated with Killed-Virus and Live-Virus Vaccines, 93.
Pigeons, Recovery of Western Equine Encephalomyelitis Virus from Crop Washings of Experimentally Infected, 139.
Turkey Lymphoid Tumor by Cellular Transplants, The Isolation and Propagation of a Naturally Occurring, 271.
Turkeys—A Comparative Study of Pleuropneumonia-like Organisms and Ornithosis in Pure and Mixed Infections, The Histopathologic Characteristics of Diseases Producing Inflammation of the Air Sacs in, 85.
Turkeys, A Study of Salmonella typhimurium Infection in Market-Age, 382.
Turkeys After 11 Weeks of Age, Evidence of Late Transmission of Staphylococcus aureus Strains Within a Divided Flock of, 753.
Turkeys with Synovitis, A Phage-Typing System for Staphylococci from, 388.

B

Bacterium anitratum (B5w) from Animals, The Recovery of, 610.
Bephenium Hydroxynaphthoate as an Anthelmintic in 8 Yearling Hereford Calves, A Critical Evaluation of, 708.
Bladder Calculi, Urine Properties, and Urethral Diameters of Feedlot Steers, Incidence of. Urolithiasis. I, 899.
Bloat—Nonvolatile Acids, The Etiology of Legume, 117.
Bloat-Producing Pastures, Biological Assays of Extracts of Alfalfa Samples from, 287.
BLOOD:
Loss Caused by Ancylostomiasis in Dogs, A Quantitative Measurement of the, 370.
of Lambs Given Preventive Treatment for White Muscle Disease, Serum Transaminase in the, 686.
Studies on Bovine Portal. II. Blood Flow Determinations with Observations on Hemodilution in the Portal Vein, 487.
Bluetongue Virus for Vaccine Preparation, The Use of Tissue Culture-Propagated, 921.
BOVINE SPECIES:
Beef Herd, Serologic Studies Suggestive of Leptospira hardjo in a, 995.
Beef Tallow, The Effect of Vitamin E Administration on Rats Fed Fresh or Autoxidized, 795.
Bovine Achondroplasia. III. Standards of Metacarpal Indexes for Achondroplastic, Brachycephalic Dwarfs and Controls, 693.
Bovine Brucellosis, A Heat-Inactivation Test for Differentiating Specific and Nonspecific Agglutination Reactions for, 564.
Bovine Enteroviruses, Studies of, 852.
Bovine Erythrocyte, Electronic Microscopy of Anaplasma marginale in the, 877.
Bovine Papular Stomatitis. II. The Experimentally Produced Disease, 473.
Bovine Papular Stomatitis. III. Histopathology, 482.
Bovine Portal Blood, Studies on. II. Blood Flow Determinations with Observations on Hemodilution in the Portal Vein, 487.
Bovine Rhinotracheitis Virus from Cattle Affected with Conjunctivitis—Observations on the Experimental Infection, The Isolation of Infectious, 13.
Bovine Serum, Polysaccharide and Protein Relationships of Normal, 986.
Bovine Staphylococcic Mastitis, Studies on. II. Characterization of Isolates, 970.
Bovine Thyroid Activity, Using Low Dosages of Radioactive Iodine I^{131}, The Measurement of, 887.
Bovine Toxoplasmosis, Neuropathology of Ovine and, 53.
Bovine Tuberculosis—Studies of Complement-Fixation Antigens, 204.
Bovine Vibrios, Antibiotic Sensitivity of, 864.
Bull Semen, Antibiotic Mediums for the Isolation of Vibrios from, 867.
Bulls, The Effects of Exogenous Testosterone on Spermatogenesis of, 209.
Calf Urine, Studies on Steroid Excretion in, 313.
Calves, A Critical Evaluation of Bephenium Hydroxynaphthoate as an Anthelmintic in 8 Yearling Hereford, 708.
Calves, A Technique for the Surgical Removal of the Forestomachs of, 990.
Calves and Adult Cattle to Bovine Anaplasmosis, The Comparative Susceptibility of, 982.
Calves and Rams, Preputial Muscles and Their Reflexes in Bull. Neural Mechanisms in Sexual Behavior. III, 37.
Calves, Development of the Causative Agent in Deer Erythrocytes Transfused into. Studies in Anaplasmosis. IV, 790.
Calves Experimentally Infected with Cooperia punctata, Growth Rate and Other Signs of Infection in, 704.
Calves, White Muscle Disease (Myopathy) in Lambs and. VII. Etiology and Prophylaxis, 466.
Cattle After Serial Passages in Chicken Embryos and Chicks, Foot-and-Mouth Disease Virus—Its Behavior in, 533.
Cattle and Horses, Vesicular Stomatitis—Patterns of Complement-Fixing and Serum-Neutralizing Antibodies in Serum of Convalescent, 713.
Cattle and Sheep, Anthelmintic Activity of an Organic Phosphate in, 893.
Cattle, Clinical and Hematologic Studies of Lymphosarcoma in a Herd of, 23.
Cattle, Horses, Sheep, and Dogs Caused by Halothane Anesthesia and the Changes Taking Place in Plasma pH and Plasma CO_2 Content, Observations on the Disturbance of Respiration of, 279.
Cattle in Northeastern Texas, Agglutinins for 11 Types of Leptospires in Serum from, 906.
Cattle Infected Naturally with Asia-1 Type Foot-and-Mouth Disease Virus, Chemical and Electrophoretic Studies of the Serums of, 217.
Cattle, Nocardia asteroides as a Mammary Pathogen of. I. The Disease in Cattle and the Comparative Virulence of 5 Isolates, 502.
Cattle, Nocardia asteroides as a Mammary Pathogen of. II. The Sources of Nocardial Infection and Experimental Reproduction of the Disease, 698.
Cattle, Studies on Shipping Fever of. II. Prevalence of Pasteurella Species in Nasal Secretions from Normal Calves and Calves with Shipping Fever, 470.
Cattle to Bovine Anaplasmosis, The Comparative Susceptibility of Calves and Adult, 982.
Cattle Treated with Diethylstilbestrol and Hexestrol, Fecal Elimination of Estrogens by, 462.
Cattle Vaccinated with HEP Flury Strain of the Virus, Colostral Antibody to Rabies in, 902.
Cows in Michigan, Quantitative Coagulase Activity and Phage Patterns of Strains of Staphylococcus aureus from, 975.
Rinderpest, A Rapid Serologic Test for, 18.
Steers, Incidence of Bladder Calculi, Urine Properties and Urethral Diameters of Feedlot. Urolithiasis. I, 899.
Steers, The Effect of Urinary Silicon Concentration on the Formation of Siliceous Deposits on Bladder Implants in, 617.
Brains of Clostridium perfringens Type D Intoxicated Lambs, Distribution of I^{131} Radioiodinated Serum Albumin in. Enterotoxemia of Sheep, 443.
Brains of Sheep and Mice, Effects of Clostridium perfringens Type D Toxin on the. Enterotoxemia of Sheep. I, 429.
BROILER—See AVIAN SPECIES
Bronchitis Vaccine, A Pasture Trial Using 2 Immunizing Doses of a Parasitic, 492.
Brucella abortus, Studies on an Immunogenic Agent Against. II. Preliminary Studies of an Immunologically Active Soluble Agglutinogen, 558.
Brucellosis, A Heat-Inactivation Test for Differentiating Specific and Nonspecific Agglutination Reactions for Bovine, 564.
BURRO—See EQUINE SPECIES

C

Calculi, Urine Properties, and Urethral Diameters of Feedlot Steers, Incidence of Bladder. Urolithiasis. I, 899.
CALF—See BOVINE SPECIES
CANINE SPECIES:
Canine Dirofilariasis, Pulmonary Arterial Lesions in, 655.
Canine Distemper Virus in the Complement-Fixation Test, Use of Cell Culture-Adapted, 1069.
Canine Heart, Further Studies on Prophylactic Therapy Against the Developing Stages of Dirofilaria immitis Before Reaching the, 1066.
Canine Hepatitis Virus Grown in Cultures of Canine Kidney Cells, Electron Microscopy of Infectious, 236.
Canine Hepatitis Virus in Tissue Culture, Cytologic Changes Induced by Infectious, 614.
Canine Neoplasms, Homologous Transplantation of, 663.
Canine Prostate Gland, The Position of the, 142.
Canine Prostatic Fluid, The Chemical Analysis of Normal, 1057.

Dog—Double Vaccination with Irradiated Larvae, Studies on Immunity to Uncinaria stenocephala Infection in the, 352.
Dog, Melanoblastic Tumors in the, 345.
Dog—Preliminary Studies, Contrast Encephalography and Ventriculography in the, 1041.
Dog, Spontaneous Abnormal Cardiac Arrhythmias and Conduction Disturbances in the. A Clinical and Pathologic Study of 3,000 Dogs, 355.
Dog, The Ages at Which the Centers of Ossification Appear Roentgenographically in the Limb Bones of the, 825.
Dogs, A Quantitative Measurement of the Blood Loss Caused by Ancylostomiasis in, 370.
Dogs, Caused by Halothane Anesthesia and the Changes Taking Place in Plasma pH and Plasma CO_2 Content, Observations on the Disturbance of Respiration of Cattle, Horses, Sheep, and, 279.
Dogs, Using Fluorscein-Labeled Antibody, Detection of Leptospires in Naturally Infected, 650.
CAPRINE SPECIES:
Goats, Establishment of Permanent Carotid Loops in (Scient. Note), 118.
Goats, Some Effects of Triflupromazine Hydrochloride on, 915.
Goats, Some Physiologic Data on, 912.
Goats, Uterine Healing After Cesarotomy in, 1097.
CARDIAC:
Arrhythmias and Conduction Disturbances in the Dog, Spontaneous Abnormal. A Clinical and Pathologic Study of 3,000 Dogs, 355.
Output and Related Values in Anesthetized Deer, 156.
Output by the Direct Fick Technique in Domestic Animals, with Observations on a Case of Traumatic Pericarditis, Estimation of, 1093.
CAT—See FELINE SPECIES
Catheterization, Exsanguination of Chickens by, 158.
CATTLE—See BOVINE SPECIES
Cell Nuclei, A Rapid, Permanent Wright's Staining Method for Chromosomes and, 807.
Cesarotomy in Goats, Uterine Healing After, 1097.
CHICK, CHICKEN—See AVIAN SPECIES
CHLORPROMAZINE:
and Ascorbic Acid in Rats During Simulated Transportation Conditions at Normal and High Temperatures, Effects of, 544.
on Heat Stress in Pigs, The Effects of, 553.
Reserpine, and Ascorbic Acid in Resisting Heat Stress in Rats, Effects of, 537.
Cholinesterase with Symptomatology in Pigs, Correlation of the Effects of Parathion on Erythrocyte, 80.
Chromosomes and Cell Nuclei, A Rapid, Permanent Wright's Staining Method for, 807.
"Cloisonné Kidney" in the Male Angora Cat, Some Histochemical Studies of, 757.
Clostridia by Crystal Violet Inhibition of Aerobic Spore-Forming Bacteria, Isolation of, 944.
Clostridium botulinum Type C Toxin, The Partial Reactivation of Formalin-Inactivated, 770.
CLOSTRIDIUM PERFRINGENS:
Type D Antitoxin Titers of Normal, Nonvaccinated Lambs. Enterotoxemia of Sheep. III, 447.
Type D Intoxicated Lambs, Distribution of I^{131} Radioiodinated Serum Albumin in Brains of. Enterotoxemia of Sheep. II, 443.
Type D Toxin on the Brains of Sheep and Mice, Effects of. Enterotoxemia of Sheep. I, 429.
Coagulase Activity and Phage Patterns of Strains of Staphylococcus aureus Isolated from Cows in Michigan, Quantitative, 975.
Coccidial Activity, A New Method for the Primary Evaluation of Anti-, 324.
Coccidioidomycosis in 3 Coyotes, 811.
Complement-Fixation Test and Wright's Stain, Demonstration of Anaplasma marginale with the Fluorescent Dye, Acridine Orange; Comparisons with the, 882.
Complement-Fixation Test, Use of Cell Culture-Adapted Canine Distemper Virus in the, 1069.
Conglutination Complement Absorption Test to Detect Hog Cholera Antibodies, Application of the. I. The Technique, 396.
Conjunctivitis—Observations on the Experimental Infection, The Isolation of Infectious Bovine Rhinotracheitis Virus from Cattle Affected with, 13.
Cooperia punctata, Growth Rate and Other Signs of Infection in Calves Experimentally Infected with, 704.
Cortisone on Leptospira pomona Infection in the Guinea Pig, Effect of, 580.
Correspondence, 624.
Coyotes, Coccidioidomycosis in 3, 811.
Creatinine Coefficient of Normal Sheep, The, 1012.
Crop in the Chicken—A Pathologic Study, Pendulous, 379.
Crystal Violet Inhibition of Aerobic Spore-Forming Bacteria, Isolation of Clostridia by, 944.
Cytopathogenic Viruses and Feline Panleukopenia Virus, Comparative Study of Feline, 374.

D

DEER:
Cardiac Output and Related Values in Anesthetized, 156.
Characteristics of the South Dakota Strain of Virus, Epizootic Hemorrhagic Disease in White-Tailed, 104.
Electron Microscopy of Anaplasma marginale in. Studies in Anaplasmosis. II, 109.
Erythrocytes Transfused into Calves, Development of the Causative Agent in. Studies in Anaplasmosis. IV, 790.
Pathology of Epizootic Hemorrhagic Disease of, 227.
Deoxycorticosterone Trimethylacetate, The in Vivo Response of Mycoplasma gallisepticum to, 749.
Dictyocaulus viviparus (Bloch), Studies on Infection and Immunity with the Lungworm. I. Active Immunization of Guinea Pigs and Rabbits, 123.
Diethylstilbestrol and Hexestrol, Fecal Elimination of Estrogens by Cattle Treated with, 462.
Diethylstilbestrol and Progesterone on the Growth of Four Mastitis-Producing Bacteria, The Effect of, 32.
Dimethoate, Against Gastrophilus spp. in the Horse, with Observations on its Anthelmintic Action, Critical Tests with the Organic Phosphate Insecticide, 1106.
Dirofilaria immitis Before Reaching the Canine Heart, Further Studies on Prophylactic Therapy Against the Developing Stages of, 1066.
Dirofilariasis, Pulmonary Arterial Lesions in Canine, 655.
Distemper Virus in the Complement-Fixation Test, Use of Cell Culture-Adapted Canine, 1069.
DUCK—See AVIAN SPECIES

E

Edema Disease of Swine, Studies on Hemolytic Escherichia coli Associated with. I. Separation and Properties of a Toxin of Hemolytic Escherichia coli, 736.
ELECTRON MICROSCOPY:
Electron Microscopic Observations of a Mucoid Strain of Vibrio fetus (Scient. Note), 1121.
of Anaplasma marginale in the Bovine Erythrocyte, 877.
of Equine Abortion Virus, 250.
of Infectious Canine Hepatitis Virus Grown in Cultures of Canine Kidney Cells, 236.
Encephalography and Ventriculography in the Dog—Preliminary Studies, Contrast, 1041.
Encephalomyelitis Virus from Crop Washings of Experimentally Infected Pigeons, Recovery of Western Equine, 139.
Encephalomyelitis Viruses as Demonstrated by the Hemagglutination-Inhibition Antibody Response of Experimentally Infected Chickens, Relationships Between Eastern and Western Equine, 266.
Endocrine-Controlled Uterine Defense Mechanism, The Leukotaxic Properties of Uterine Exudates as Related to the, 1117.
ENTEROTOXEMIA:
of Sheep. I. Effects of Clostridium perfringens Type D Toxin on the Brains of Sheep and Mice, 429.
of Sheep. II. Distribution of I^{131} Radiolodinated Serum Albumin in Brains of Clostridium perfringens Type D Intoxicated Lambs, 443.
of Sheep. III. Clostridium perfringens Type D Antitoxin Titers of Normal, Nonvaccinated Lambs, 447.
Enteroviruses, Studies of Bovine, 852.
Enzyme Activity Levels in Chicken Plasma, 99.
EQUINE SPECIES:
Burro, The Protozoan Parasite Klossiella equi in the Mexican (Scient. Note), 1126.
Equine Abortion Virus, Electron Microscopy of, 250.
Equine Animals, Intravenous Ether Anesthesia in, 720.
Equine Arteritis Virus in Monolayer Cultures of Equine Kidney, Propagation of, 731.
Equine Encephalomyelitis Virus from Crop Washings of Experimentally Infected Pigeons, Recovery of Western, 139.
Equine Encephalomyelitis Viruses as Demonstrated by the Hemagglutination-Inhibition Antibody Response of Experimentally Infected Chickens, Relationships Between Eastern and Western, 266.
Horse, Some Quantitative and Pathologic Aspects of Natural and Experimental Infections in the. Studies on Trichostrongylus axei (Cobbold, 1879). VII, 128.
Horse, with Observations on its Anthelmintic Action, Critical Tests with the Organic Phosphate Insecticide, Dimethoate, Against Gastrophilus spp. in the, 1106.
Horses, Sheep, and Dogs Caused by Halothane Anesthesia and the Changes Taking Place in Plasma pH and Plasma CO_2 Content, Observations on the Disturbance of Respiration of Cattle, 279.
Horses, Vesicular Stomatitis—Patterns of Complement-Fixing and Serum-Neutralizing Antibodies in Serum of Convalescent Cattle and, 713.

ERYSIPELOTHRIX RHUSIOPATHIAE:
The Antigenic Structure of Virulent and Avirulent Strains of. I. Immunobiologic Properties, 836.
The Antigenic Structure of Virulent and Avirulent Strains of. II. Immunochemic and Serologic Investigations, 839.
The Antigenic Structure of Virulent and Avirulent Strains of. III. The Agar Gel Diffusion-Precipitation Test, 846.
ERYTHROCYTE:
Cholinesterase with Symptomatology in Pigs, Correlation of the Effects of Parathion on, 80.
Electron Microscopy of Anaplasma marginale in the Bovine, 877.
Sedimentation Rate in Domestic Animals, Studies on the. VII. Rouleau Formation, 319.
Survival in Experimental Anaplasmosis, 590.
Survival Studies in Domestic and Bighorn Sheep, Using Glycine-2-C^{14}, 683.
Transfused into Calves, Development of the Causative Agent in Deer. Studies in Anaplasmosis. IV, 790.
Escherichia coli Associated with Edema Disease of Swine, Studies on Hemolytic. I. Separation and Properties of a Toxin of Hemolytic Escherichia coli, 736.
Estrogens by Cattle Treated with Diethylstilbestrol and Hexestrol, Fecal Elimination of, 462.
Estrous Cycle, Variation in the Inflammatory Response and Bactericidal Activity of the Sheep Uterus During the, 689.
Ether Anesthesia in Equine Animals, Intravenous, 720.
Ethylene Oxide on Dried Foot-and-Mouth Disease Virus, The Effect of Gaseous, 779.
Exsanguination of Chickens by Catheterization, 158.

F

(Fasciolopsis buski) in a Monkey, The Intestinal Fluke (Scient. Note), 1123.
Fat for Intraperitoneal Alimentation, Use of Emulsified, 147.
Fecal Elimination of Estrogens by Cattle Treated with Diethylstilbestrol and Hexestrol, 462.
FELINE SPECIES:
Cat, Some Histochemical Studies of "Cloisonné Kidney" in the Male Angora, 757.
Cats, Studies on Meningiomas in, 1030.
Feline Cytopathogenic Viruses and Feline Panleukopenia Virus, Comparative Study of, 374.
Feline Virus Isolated by Bolin to Feline Panleukopenia, Kidney Cell Degenerating Virus, and 2 Feline Respiratory Viruses, The Relationship of a, 637.
Fick Technique in Domestic Animals, with Observations on a Case of Traumatic Pericarditis, Estimation of Cardiac Output by the Direct, 1093.
Flocculation Test for the Detection of Antibodies Resulting from the Injection of an Extract of Hypoderma Larvae, A, 332.
(Florent) from Sheep, The Isolation of Vibrio bubulus, 449.
Fluorescein-Labeled Antibody, Detection of Leptospires in Naturally Infected Dogs, Using, 650.
Fluorescein-Labeled Antibody Technique for Identification of Leptospiral Serotypes, 570.
Fluorescent Dye, Acridine Orange: Comparisons with the Complement-Fixation Test and Wright's Stain, Demonstration of Anaplasma marginale with the, 882.
FOOT-AND-MOUTH DISEASE VIRUS:
Chemical and Electrophoretic Studies of the Serums of Cattle Infected Naturally with Asia-1 Type, 217.
The Effect of Gaseous Ethylene Oxide on Dried, 779.
in Cultures of Cells on Glass, The Production of High Concentrations of, 926.
in Meat, Further Studies on the Survival of, 224.
Its Behavior in Cattle After Serial Passages in Chicken Embryos and Chicks, 533.
I. Propagation of 3 Immunologic Types of Virus in Chicks, 518.
II. Propagation and Modification of 3 Immunologic Types of Virus in Embryonating Chicken Eggs, 527.
with High Complement-Fixing Antigenicity, Production of, 775.

G

Gallbladder in Pigs with Hog Cholera, Focal Necrosis in the Mucosa of the, 391.
Gamma Radiation on Testicular Morphology and Function in Swine, Effects of Prenatal, 406.
Gastrophilus spp. in the Horse, with Observations on Its Anthelmintic Action, Critical Tests with the Organic Phosphate Insecticide, Dimethoate, Against, 1106.
Gel Diffusion Technique, Study of Polysaccharide-Antibody Reactions by a Rapid. Characterization of Vibrio fetus Antigens. IV, 783.
Glucose Oxidase, Estimation of Low Concentrations of Plasma Glucose Using, 587.
Glycine-2-C^{14}, Erythrocyte Survival Studies in Domestic and Bighorn Sheep, Using, 683.
GOAT—See CAPRINE SPECIES
Growth of Laboratory Animals, Effect of Naturally Occurring Highly Mineralized Water on the, 601.
Growth Rate and Other Signs of Infection in Calves Experimentally Infected with Cooperia punctata, 704.
GUINEA PIG—See LABORATORY ANIMALS

H

Haemonchus contortus Infection—Double Vaccination of Sheep with Irradiated Larvae, Studies on Immunity to, 186.
Halothane Anesthesia and the Changes Taking Place in Plasma pH and Plasma CO_2 Content, Observations on the Disturbance of Respiration of Cattle, Horses, Sheep, and Dogs Caused by, 279.
HAMSTER—See LABORATORY ANIMALS
HEAT:
Inactivation Test for Differentiating Specific and Nonspecific Agglutination Reactions for Bovine Brucellosis, A, 564.
Stress in Pigs, The Effects of Chlorpromazine on, 553.
Stress in Rats, Effects of Chlorpromazine, Reserpine, and Ascorbic Acid in Resisting, 537.
Stress in the Rat, Thyroid-Active Agents and, 549.
Helminths of Market Weight Pigs, The Incidence of Some Gastrointestinal. Parasites of Domestic Animals in Indiana. I, 746.
Hemagglutination-Inhibition Antibody Response of Experimentally Infected Chickens, Relationships Between Eastern and Western Equine Encephalomyelitis Viruses as Demonstrated by the, 266.
Hemolytic Escherichia coli Associated with Edema Disease of Swine, Studies on. I. Separation and Properties of a Toxin of Hemolytic Escherichia coli, 736.
Hemorrhagic Disease in White-Tailed Deer—Characteristics of the South Dakota Strain of Virus, Epizootic, 104.
Hemorrhagic Disease of Deer, Pathology of Epizootic, 227.
HEP Flury Strain of the Virus. Colostral Antibody to Rabies in Cattle Vaccinated with, 902.
HEPATITIS:
A Modified Microgel Diffusion Method and Its Application in the Study of the Virus of Duck, 274.
Virus Grown in Cultures of Canine Kidney Cells, Electron Microscopy of Infectious Canine, 236.
Virus in Tissue Culture, Cytologic Changes Induced by Infectious Canine, 614.
Herpes B Virus, Preliminary Findings Concerning the Formalin Inactivation of, 1112.
Hexestrol, Fecal Elimination of Estrogens by Cattle Treated with Diethylstilbestrol and, 462.
HORSE—See EQUINE SPECIES
(Hymenoxys richardsonii) Poisoning in Sheep, Pingue, 47.
Hypoderma Larvae, A Flocculation Test for the Detection of Antibodies Resulting from the Injection of an Extract of, 332.

I

Ileitis in Rats—A Report of 64 Cases, Spontaneous, 932.
Immunization Against Ovine Vibriosis, Experimental. I. The Use of Live and Formalin-Killed Vibrio fetus Vaccines, 43.
Inoculation of Microbes into the Intestinal Tracts of Small Animals, 330.
Insecticide, Dimethoate, Against Gastrophilus spp. in the Horse, with Observations on Its Anthelmintic Action, Critical Tests with the Organic Phosphate, 1106.
INTESTINAL:
Fluke (Fasciolopsis buski) in a Monkey, The (Scient. Note), 1123.
Mucosa, A Technique for Concentrating Mycobacterium paratuberculosis Present in, 1074.
Tracts of Small Animals, Inoculation of Microbes into the, 330.
Iodine^{131}, The Measurement of Bovine Thyroid Activity, Using Low Dosages of Radioactive, 887.

J

Johne's Disease in Sheep, Studies on the Pathology of. III. Pathologic Changes in Sheep with the Naturally Occurring Infections, 189.

K

Ketosis, Metabolic Aspects of Ruminant, 496.
KIDNEY:
Cell Degenerating Virus, and 2 Feline Respiratory Viruses, The Relationship of a Feline Virus Isolated by Bolin to Feline Panleukopenia, 637.
Cells, Electron Microscopy by Infectious Canine Hepatitis Virus Grown in Cultures of Canine, 236.
"Cloisonné Kidney" in the Male Angora Cat, Some Histochemical Studies of, 757.
Propagation of Equine Arteritis Virus in Monolayer Cultures of Equine, 731.
Klossiella equi in the Mexican Burro, The Protozoan Parasite (Scient. Note), 1126.

L

LABORATORY ANIMALS:
Guinea Pig, Effect of Cortisone on Leptospira pomona Infection in the, 580.
Guinea Pigs and Rabbits, Active Immunization of. Studies on Infection and Immunity with the Lungworm, Dictyocaulus viviparus (Bloch), I, 123.
Guinea Pigs, Direct Cultural Methods for the Isolation of Leptospires from Experimentally Infected, 1085.
Hamsters on Diets Containing Various Levels of Riboflavin, Leptospirosis in, 800.
Laboratory Animals, Effect of Naturally Occurring Highly Mineralized Water on the Growth of, 601.
Mice, Effects of Clostridium perfringens Type D Toxin on the Brains of Sheep and. Enterotoxemia of Sheep. I, 429.
Monkey, The Intestinal Fluke (Fasciolopsis buski) in a (Scient. Note), 1123.
Mouse Colonies, Oxyuriasis in Laboratory, 150.
Rabbits, Active Immunization of Guinea Pigs and. Studies on Infection and Immunity with the Lungworm, Dictyocaulus viviparus (Bloch). I, 123.
Rat, Thyroid-Active Agents and Heat Stress in the, 549.
Rats—A Report of 64 Cases, Spontaneous Ileitis in, 932.
Rats During Simulated Transportation Conditions at Normal and High Temperatures, Effects of Chlorpromazine and Ascorbic Acid in, 544.
Rats, Effects of Chlorpromazine, Reserpine, and Ascorbic Acid in Resisting Heat Stress in, 537.
Rats Fed Fresh or Autoxidized Beef Tallow, The Effect of Vitamin E Administration on, 795.
LAMBS—See OVINE SPECIES
LEPTOSPIRA:
hardjo in a Beef Herd, Serologic Studies Suggestive of, 995.
pomona in Composite Herd Milk Samples, The Detection of Antibody Against, 573.
pomona Infection in the Guinea Pig, Effect of Cortisone on, 580.
Serotypes, Fluorescein-Labeled Antibody Technique for Identification of, 570.
LEPTOSPIRES:
from Experimentally Infected Guinea Pigs, Direct Cultural Methods for the Isolation of, 1085.
in Naturally Infected Dogs, Using Fluorescein-Labeled Antibody, Detection of, 650.
in Serum from Cattle in Northeastern Texas, Agglutinins for 11 Types of, 906.
The Use of Agar Plates as an Aid for the Isolation of, 306.
LEPTOSPIROSIS:
in Hamsters on Diets Containing Various Levels of Riboflavin, 800.
in the Meadow Vole, Microtus pennsylvanicus, Natural Occurrence of (Scient. Note), 949.
Studies on a Skin Test for, 1081.
Leukotaxic Properties of Uterine Exudates as Related to the Endocrine-Controlled Uterine Defense Mechanism, The, 1117.
Liver, Characterization of 2 Viruses Obtained from Lymphomatous, 937.
Livestock Toxic Disease, Oxalate Formation in Moldy Feedstuffs as a Possible Factor in, 961.
Lungs, Microbiologic Survey of Pneumonic and Normal Swine, 1020.
Lungworm, Dictyocaulus viviparus (Bloch), Studies on Infection and Immunity with the. I. Active Immunization of Guinea Pigs and Rabbits, 123.
Lymphomatous Liver, Characterization of 2 Viruses Obtained from, 937.
Lymphosarcoma in a Herd of Cattle, Clinical and Hematologic Studies of, 23.

M

Mastitis-Producing Bacteria, The Effect of Diethylstilbestrol and Progesterone on the Growth of Four, 32.
Mastitis, Studies on Bovine Staphylococcic. II. Characterization of Isolates, 970.
Melanoblastic Tumors in the Dog, 345.
Meningiomas in Cats, Studies on, 1030.
Metastrongylosis in the Field: Species and Sex Ratios of the Parasites, Prefential Location in Respiratory Apparatus of the Host, and Concomitant Lesions, 606.
Metastrongylus and Ascaris Eggs in 3 Different Levitation Solutions, Comparison of the Flotation of, 597.
Metastrongylus Molin, 1861 (Nematoda: Metastrongylidae), Association Among Members of the Genus, 1077.
Mexican Burro, The Protozoan Parasite Klossiella equi in the (Scient. Note), 1126.
Microbes Into the Intestinal Tracts of Small Animals, Inoculation of, 330.
Microgel Diffusion Method and Its Application in the Study of the Virus of Duck Hepatitis, a Modified, 274.
Microtus pennsylvanicus, Natural Occurrence of Leptospirosis in the Meadow Vole (Scient. Note), 949.
Mineralized Water on the Growth of Laboratory Animals, Effect of Naturally Occurring Highly, 601.
Moldy Feedstuffs as a Possible Factor in Livestock Toxic Disease, Oxalate Formation in, 961.
MOUSE—See LABORATORY ANIMALS
MUSCULAR DYSTROPHY:
in Lambs—Administration of Selenium to Affected and Unaffected Lambs, Nutritional, 416.
in Lambs—Effect of Administering Selenium to Pregnant Ewes, Nutritional, 419.
in Lambs—Importance of the Ewe's Diet at Certain Stages of Gestation and Lactation, Nutritional, 412.
Mycobacterium paratuberculosis Present in Intestinal Mucosa, A Technique for Concentrating, 1074.
Mycoplasma gallisepticum to Deoxycorticosterone Trimethylacetate, The in Vivo Response of, 749.
(Myopathy) in Lambs and Calves, White Muscle Disease, VII. Etiology and Prophylaxis, 466.

N

Nasal Virus in Experimental Pigs, Site of Localization and Effects of Swine, 67.
Necrosis in the Mucosa of the Gallbladder in Pigs with Hog Cholera, Focal, 391.
(Nematoda: Metastrongylidae), Association Among Members of the Genus Metastrongylus Molin, 1861, 1077.
Neoplasms, Homologous Transplantation of Canine, 663.
NEWCASTLE DISEASE—See AVIAN SPECIES
Nocardia asteroides as a Mammary Pathogen of Cattle. I. The Disease in Cattle and the Comparative Virulence of 5 Isolates, 502.
Nocardia asteroides as a Mammary Pathogen of Cattle. II. The Sources of Nocardial Infection and Experimental Reproduction of the Disease, 698.
NUTRITIONAL:
Muscular Dystrophy in Lambs—Administration of Selenium to Affected and Unaffected Lambs, 416.
Muscular Dystrophy in Lambs—Effect of Administering Selenium to Pregnant Ewes, 419.
Muscular Dystrophy in Lambs—Importance of the Ewe's Diet at Certain Stages of Gestation and Lactation, 412.

O

Ornithosis in Pure and Mixed Infections, The Histopathologic Characteristics of Diseases Producing Inflammation of the Air Sacs in Turkeys—A Comparative Study of Pleuropneumonia-like Organisms and, 85.
Ossification Appear Roentgenographically in the Limb Bones of the Dog, The Ages at Which the Centers of, 825.
OVINE SPECIES:
Lambs—Administration of Selenium to Affected and Unaffected Lambs, Nutritional Muscular Dystrophy in, 416.
Lambs and Calves, White Muscle Disease (Myopathy) in. VII. Etiology and Prophylaxis, 466.
Lambs—Effect of Administering Selenium to Pregnant Ewes, Nutritional Muscular Dystrophy in, 419.
Lambs, Factors Influencing the incidence of Chronic Pneumonia in, 1015.
Lambs Given Preventive Treatments for White Muscle Disease, Serum Transaminase in the Blood of, 686.
Lambs—Importance of the Ewe's Diet at Certain Stages of Gestation and Lactation, Nutritional Muscular Dystrophy in, 412.
Ovine and Bovine Toxoplasmosis, Neuropathology of, 53.
Rams, Preputial Muscles and Their Reflexes in Bull Calves and. Neural Mechanisms in Sexual Behavior. III, 37.

Sheep, and Dogs Caused by Halothane Anesthesia and the Changes Taking Place in the Plasma pH and Plasma CO_2 Content, Observations on the Disturbance of Respiration of Cattle, Horses, 279.
Sheep, Anthelmintic Activity of an Organic Phosphate in Cattle and, 893.
Sheep, Enterotoxemia of. I. Effects of Clostridium perfringens Type D Toxin on the Brains of Sheep and Mice, 429.
Sheep, Enterotoxemia of. II. Distribution of I^{131} Radioiodinated Serum Albumin in Brains of Clostridium perfringens Type D Intoxicated Lambs, 443.
Sheep, Enterotoxemia of. III. Clostridium perfringens Type D Antitoxin Titers of Normal, Nonvaccinated Lambs, 447.
Sheep—Metastasizing Bronchiolar Tumors, Pulmonary Adenomatosis of, 673.
Sheep, Pingue (Hymenoxys richardsonii) Poisoning in, 47.
Sheep, Serum and Tissue Residues Following Selenium Injections in, 422.
Sheep, Studies on the Pathology of Johne's Disease in. III. Pathologic Changes in Sheep with Naturally Occurring Infections, 189.
Sheep, The Creatinine Coefficient of Normal, 1012.
Sheep, The Isolation of Vibrio bubulus (Florent) from, 449.
Sheep, Ultrafiltration, Electrophoretic and Chromatographic Studies on Urinary Biocolloids of. Ruminant Urolithiasis. IV, 1000.
Sheep, Using Glycine-2-C^{14}, Erythrocyte Survival Studies in Domestic and Bighorn, 685.
Sheep Uterus During the Estrous Cycle, Variation in the Inflammatory Response and Bactericidal Activity of the, 689.
Sheep with Irradiated Larvae, Studies on Immunity to Haemonchus contortus Infection—Double Vaccination of, 186.
Sheep with Vibriosis, Placental Pathology of, 169.
Oxalate Formation in Moldy Feedstuffs as a Possible Factor in Livestock Toxic Disease, 961.
Oxyuriasis in Laboratory Mouse Colonies, 150.

P

Panleukopenia, Kidney Cell Degenerating Virus, and 2 Feline Respiratory Viruses, The Relationship of a Feline Virus Isolated by Bolin to Feline, 637.
Panleukopenia Virus, Comparative Study of Feline Cytopathogenic Viruses and Feline, 374.
Papular Stomatitis, Bovine. II. The Experimentally Produced Disease, 473.
Papular Stomatitis, Bovine. III. Histopathology, 482.
Parasite Klossiella equi in the Mexican Burro, The Protozoan (Scient. Note), 1126.
Parasites of Domestic Animals in Indiana. I. The Incidence of Some Gastrointestinal Helminths of Market Weight Pigs, 746.
Parasitic Bronchitis Vaccine, A Pasture Trial Using 2 Immunizing Doses of a, 492.
Parathion on Erythrocyte Cholinesterase with Symptomatology in Pigs, Correlation of the Effects of, 80.
Pasteurella Species in Nasal Secretions from Normal Calves and Calves with Shipping Fever, Prevalence of. Studies on Shipping Fever of Cattle. II, 470.
Pasture Trial Using 2 Immunizing Doses of a Parasitic Bronchitis Vaccine, A, 492.
Pastures, Biological Assays of Extracts of Alfalfa Samples from Bloat-Producing, 287.
Pericarditis, Estimation of Cardiac Output by the Direct Fick Technique in Domestic Animals, with Observations on a Case of Traumatic, 1093.
Phage-Typing System for Staphylococci from Turkeys with Synovitis, A, 388.
Phosphate in Cattle and Sheep, Anthelmintic Activity of an Organic, 893.
Phosphate Insecticide, Dimethoate, Against Gastrophilus spp. in the Horse, with Observations on Its Anthelmintic Action, Critical Tests with the Organic, 1106.
PIGEONS—See AVIAN SPECIES
Pingue (Hymenoxys richardsonii) Poisoning in Sheep, 47.
Placental Pathology of Sheep with Vibriosis, 169.
Plasma Glucose Using Glucose Oxidase, Estimation of Low Concentrations of, 587.
Plasma pH and Plasma CO_2 Content, Observations on the Disturbance of Respiration of Cattle, Horses, Sheep, and Dogs Caused by Halothane Anesthesia and the Changes Taking Place in, 279.
Pleuropneumonia-like Organisms and Ornithosis in Pure and Mixed Infections, The Histopathologic Characteristics of Diseases Producing Inflammation of the Air Sacs in Turkeys—A Comparative Study of, 85.
Pneumonia in Lambs, Factors Influencing the Incidence of Chronic, 1015.
Poisoning in Sheep, Pingue (Hymenoxys richardsonii), 47.
Polysaccharide and Protein Relationships of Normal Bovine Serum, 986.
Polysaccharide-Antibody Reactions by a Rapid Slide Gel Diffusion Technique, Study of. Characterization of Vibrio fetus Antigens. IV, 783.
PORCINE SPECIES:
Hog Cholera Antibodies, Application of the Conglutination Complement Absorption Test to Detect. I. The Technique, 396.
Hog Cholera, Focal Necrosis in the Mucosa of the Gallbladder in Pigs with, 391.
Hog Cholera Virus in Subculturable Swine Buffy Coat Cells, Cultivation of, 741.
Pigs, Correlation of the Effects of Parathion on Erythrocyte Cholinesterase with Symptomatology in, 80.
Pigs, Post-Tetanic Potentiation in Spinal Cord of Normal and Myoclonic, 72.
Pigs, The Effects of Chlorpromazine on Heat Stress in, 553.
Pigs, The Incidence of Some Gastrointestinal Helminths of Market Weight. Parasites of Domestic Animals in Indiana. I, 746.
Pigs with Hog Cholera, Focal Necrosis in the Mucosa of the Gallbladder in, 391.
Swine, Effects of Prenatal Gamma Radiation on Testicular Morphology and Function in, 406.
Swine Lungs, Microbiologic Survey of Pneumonic and Normal, 1020.
Swine Nasal Virus in Experimental Pigs, Site of Localization and Effects of, 67.
Swine, Serum Transaminase and Aldolase During Migration of Larval Ascaris suum in, 1026.
Swine, Studies on Hemolytic Escherichia coli Associated with Edema Disease of. I. Separation and Properties of a Toxin of Hemolytic Escherichia coli, 736.
Swine, Toxicity of Wood Preservatives for, 401.
Portal Blood, Studies on Bovine. II. Blood Flow Determinations with Observations on Hemodilution in the Portal Vein, 487.
Preputial Cavity in Domestic Animals, Mode of Formation of the, 764.
Progesterone on the Growth of Four Mastitis-Producing Bacteria, The Effect of Diethylstilbestrol and, 32.
Prostate Gland, The Position of the Canine, 142.
Prostatic Fluid, The Chemical Analysis of Normal Canine, 1057.
Protein Relationships of Normal Bovine Serum, Polysaccharide and, 986.
Protozoan Parasite Klossiella equi in the Mexican Burro, The (Scient. Note), 1126.
Pulmonary Adenomatosis of Sheep—Metastasizing Bronchiolar Tumors, 673.
Pulmonary Arterial Lesions in Canine Dirofilariasis, 655.

R

RABBIT—See LABORATORY ANIMALS
Rabies in Cattle Vaccinated with HEP Flury Strain of the Virus, Colostral Antibody to, 902.
Rabies Vaccines, Potency Testing of Low Egg Passage Modified Live-Virus, 644.
Radioactive Iodine^{131}, The Measurement of Bovine Thyroid Activity, Using Low Dosages of, 887.
(Radiography) The Ages at Which the Centers of Ossification Appear Roentgenographically in the Limb Bones of the Dog, 825.
Radioiodinated Serum Albumin in Brains of Clostridium perfringens Type D Intoxicated Lambs, Distribution of I^{131}, Enterotoxemia of Sheep. II, 443.
RAM—See OVINE SPECIES
RAT—See LABORATORY ANIMALS
Reserpine, and Ascorbic Acid in Resisting Heat Stress in Rats, Effects of Chlorpromazine, 537.
Respiration of Cattle, Horses, Sheep, and Dogs Caused by Halothane Anesthesia and the Changes Taking Place in Plasma pH and Plasma CO_2 Content, Observations on the Disturbance of, 279.
Respiratory Viruses, The Relationship of a Feline Virus Isolated by Bolin to Feline Panleukopenia Kidney Cell Degenerating Virus, and 2 Feline, 637.
Rhinotracheitis Virus from Cattle Affected with Conjunctivitis—Observations on the Experimental Infection, The Isolation of Infectious Bovine, 13.
Riboflavin, Leptospirosis in Hamsters on Diets Containing Various Levels of, 800.
RINDERPEST—See BOVINE SPECIES
Rouleau Formation. Studies on the Erythrocyte Sedimentation Rate in Domestic Animals. VII, 319.
Ruminant Ketosis, Metabolic Aspects of, 496.
Ruminant Urolithiasis. IV. Ultrafiltration, Electrophoretic and Chromatographic Studies on Urinary Biocolloids of Sheep, 1000.

S

Salmonella typhimurium Infection in Market-Age Turkeys, A Study of, 382.
Sarcoma (Strain 13) by Vent Exposure, Study of Chicken, 813.
Sarcoma (Strain 13) Placed in the Bursa of Fabricius, Study of Chicken, 154.
SCIENTIFIC NOTES:
Cardiac Output and Related Values in Anesthetized Deer, 156.
Coccidioidomycosis in 3 Coyotes, 811.
Cytologic Changes Induced by Infectious Canine Hepatitis Virus in Tissue Culture, 614.
The Effect of Urinary Silicon Concentration on the Formation of Siliceous Deposits on Bladder Implants in Steers, 617.
Electron Microscopic Observations of a Mucoid Strain of Vibrio fetus, 1121.
Establishment of Permanent Carotid Loops in Goats, 948.
Exsanguination of Chickens by Catheterization, 158.
A Flocculation Test for the Detection of Antibodies Resulting from the Injection of an Extract of Hypoderma Larvae, 332.
Inoculation of Microbes into the Intestinal Tracts of Small Animals, 330.
The Intestinal Fluke (Fasciolopsis buski) in a Monkey, 1123.
Natural Occurrence of Leptospirosis in the Meadow Vole, Microtus pennsylvanicus, 949.
The Protozoan Parasite Klossiella equi in the Mexican Burro, 1126.
Study of Chicken Sarcoma (Strain 13) by Vent Exposure, 813.
Study of Chicken Sarcoma (Strain 13) Placed in the Bursa of Fabricius, 154.
SELENIUM:
Injections in Sheep, Serum and Tissue Residues Following, 422.
to Affected and Unaffected Lambs, Nutritional Muscular Dystrophy in Lambs—Administration of, 416.
to Pregnant Ewes, Nutritional Muscular Dystrophy in Lambs—Effect of Administering, 419.
Semen, Antibiotic Mediums for the Isolation of Vibrios from Bull, 867.
Serologic Studies Suggestive of Leptospira hardjo in a Beef Herd, 995.
SERUM:
and Tissue Residues Following Selenium Injections in Sheep, 422.
of Cattle Infected Naturally with Asia-1 Type Foot-and-Mouth Disease Virus, Chemical and Electrophoretic Studies of the, 217.
Polysaccharide and Protein Relationships of Normal Bovine, 986.
Transaminase and Aldolase During Migration of Larval Ascaris suum in Swine, 1026.
Transaminase in the Blood of Lambs Given Preventive Treatments for White Muscle Disease, 686.
Sexual Behavior, Neural Mechanisms in. III. Preputial Muscles and Their Reflexes in Bull Calves and Rams, 37.
Shipping Fever of Cattle. Studies on. II. Prevalence of Pasteurella Species in Nasal Secretions from Normal Calves and Calves with Shipping Fever, 470.
Silicon Concentration on the Formation of Siliceous Deposits on Bladder Implants in Steers, The Effect of Urinary (Scient. Note), 617
Skin Test for Leptospirosis, Studies on a, 1081.
South Dakota Strain of Virus, Epizootic Hemorrhagic Disease in White-Tail Deer—Characteristics of the, 104.
Spermatogenesis of Bulls, The Effects of Exogenous Testosterone on, 209.
Staphylococci from Turkeys with Synovitis, A Phage-Typing System for, 388.
Staphylococcic Mastitis, Studies on Bovine. II. Characterization of Isolates, 970.
Staphylococcus aureus Isolated from Cows in Michigan, Quantitative Coagulase Activity and Phage Patterns of Strains of, 975.
Staphylococcus aureus Strains Within a Divided Flock of Turkeys After 11 Weeks of Age, Evidence of Late Transmission of, 753.
Steroid Excretion in Calf Urine, Studies on, 313.
(Stomach) A Technique for the Surgical Removal of the Forestomachs of Calves, 990.
STOMATITIS:
Bovine Papular. II. The Experimentally Produced Disease, 473.
Bovine Papular. III. Histopathology, 482.
Patterns of Complement-Fixing and Serum-Neutralizing Antibodies in Serum of Convalescent Cattle and Horses, Vesicular, 713.
SWINE—See PORCINE SPECIES
Synovitis, A Phage-Typing System for Staphylococci from Turkeys with, 388.

T

Testicular Morphology and Function in Swine, Effects of Prenatal Gamma Radiation on, 406.
Testosterone on Spermatogenesis of Bulls, The Effects of Exogenous, 209.
(Tetanus) Post-Tetanic Potentiation in Spinal Cord of Normal and Myoclonic Pigs, 72.
Thyroid-Active Agents and Heat Stress in the Rat, 549.
Thyroid Activity, Using Low Dosages of Radioactive Iodine I^{131}, The Measurement of Bovine, 887.
Toxic Disease, Oxalate Formation in Moldy Feedstuffs as a Possible Factor in Livestock, 961.
Toxicity of Wood Preservatives for Swine, 401.
Toxoplasmosis, Neuropathology of Ovine and Bovine, 53.
Transplantation of Canine Neoplasms, Homologous, 663.
Trichostrongylus axei (Cobbold, 1879), Studies on. VII. Some Quantitative and Pathologic Aspects of Natural and Experimental Infections in the Horse, 128.
Triflupromazine Hydrochloride on Goats, Some Effects of, 915.
Tuberculosis—Studies of Complement-Fixation Antigens, Bovine, 204.
TUMOR:
by Cellular Transplants, The Isolation and Propagation of a Naturally Occurring Turkey Lymphoid, 271.
in the Dog, Melanoblastic, 345.
Pulmonary Adenomatosis of Sheep—Metastasizing Bronchiolar, 673.
TURKEY—See AVIAN SPECIES

U

Uncinaria stenocephala Infection in the Dog—Double Vaccination with Irradiated Larvae, Studies on Immunity to, 352.
Urinary Silicon Concentration on the Formation of Siliceous Deposits on Bladder Implants in Steers, The Effect of (Scient. Notes), 617.
Urine, Studies on Steroid Excretion in Calf, 313.
Urolithiasis. I. Incidence of Bladder Calculi, Urine Properties, and Urethral Diameters of Feedlot Steers, 899.
Urolithiasis, Ruminant. IV. Ultrafiltration, Electrophoretic and Chromatographic Studies on Urinary Biocolloids of Sheep, 1000.
Uterine Exudates as Related to the Endocrine-Controlled Uterine Defense Mechanism, The Leukotaxic Properties of, 1117.
Uterine Healing After Cesarotomy in Goats, 1097.
Uterus During the Estrous Cycle, Variation in the Inflammatory Response and Bactericidal Activity of the Sheep, 689.

V

VACCINE:
Newcastle Disease Immunity in Broilers and Capons vaccinated with Killed-Virus and Live-Virus, 93.
A Pasture Trial Using 2 Immunizing Doses of a Parasitic Bronchitis, 492.
Potency Testing of Low Egg Passage Modified Live-Virus Rabies, 644.
Preparation, The Use of Tissue Culture-Propagated Bluetongue Virus for, 921.
The Use of Live and Formalin-Killed Vibrio fetus. Experimental Immunization Against Ovine Vibriosis. I. 43.
Ventriculography in the Dog—Preliminary Studies, Contrast Encephalography and, 1041.
Vesicular Stomatitis—Patterns of Complement-Fixing and Serum-Neutralizing Antibodies in Serum of Convalescent Cattle and Horses, 713.
VIBRIO:
Antibiotic Sensitivity of Bovine, 864.
bubulus (Florent) from Sheep, The Isolation of, 449.
fetus Antigens, Characterization of. IV. Study of Polysaccharide-Antibody Reactions by a Rapid Slide Gel Diffusion Technique, 783.
fetus, Electron Microscopic Observations of a Mucoid Strain of (Scient. Note), 1121.
fetus Isolation in Vitro Study of Extracted Antigens of, 327.
fetus Vaccines, The Use of Live and Formalin-Killed. Experimental Immunization Against Ovine Vibriosis. I. 43.
Vibriosis, Experimental Immunization Against Ovine. I. The Use of Live and Formalin-Killed Vibrio fetus Vaccines, 43.

Vibriosis, Placental Pathology of Sheep with, 169.
VIRUS:
as Demonstrated by the Hemagglutination-Inhibition Antibody Response of Experimentally Infected Chickens, Relationships Between Eastern and Western Equine Encephalomyelitis, 266.
Chemical and Electrophoretic Studies of the Serums of Cattle Infected Naturally with Asia-1 Type Foot-and-Mouth Disease, 217.
Colostral Antibody to Rabies in Cattle Vaccinated with HEP Flury Strain of the, 902.
Comparative Study of Feline Cytopathogenic Viruses and Feline Panleukopenia, 374.
The Effect of Gaseous Ethylene Oxide on Dried Foot-and-Mouth Disease, 779.
Electron Microscopy of Equine Abortion, 250.
Foot-and-Mouth Disease. I. Propagation of 3 Immunologic Types of Virus in Chicks, 518.
Foot-and-Mouth Disease. II. Propagation and Modification of 3 Immunologic Types of Virus in Embryonating Chicken Eggs, 527.
for Vaccine Preparation, The Use of Tissue Culture-Propagated Bluetongue, 921.
from Cattle Affected with Conjunctivitis—Observations on the Experimental Infection, The Isolation of Infectious Bovine Rhinotracheitis, 13.
from Crop Washings of Experimentally Infected Pigeons, Recovery of Western Equine Encephalomyelitis, 139.
Grown in Cultures of Canine Kidney Cells, Electron Microscopy of Infectious Canine Hepatitis, 236.
in Cultures of Cells on Glass, The Production of High Concentrations of Foot-and-Mouth Disease, 926.
in Experimental Pigs, Site of Localization and Effects of Swine Nasal, 67.
in Meat, Further Studies on the Survival of Foot-and-Mouth Disease, 224.
in Monolayer Cultures of Equine Kidney, Propagation of Equine Arteritis, 731.
in Subculturable Swine Buffy Coat Cells, Cultivation of Hog Cholera, 741.
in the Complement-Fixation Test, Use of Cell Culture-Adapted Canine Distemper, 1069.
in Tissue Culture, Cytologic Changes Induced by Infectious Canine Hepatitis, 614.
Isolated by Bolin to Feline Panleukopenia, Kidney Cell Degenerating Virus, and 2 Feline Respiratory Viruses, The Relationship of a Feline, 637.
Its Behavior in Cattle After Serial Passages in Chicken Embryos and Chicks, Foot-and-Mouth Disease, 533.
Obtained from Lymphomatous Liver, Characterization of 2, 937.
of Duck Hepatitis, A Modified Microgel Diffusion Method and Its Application in the Study of the, 274.
Preliminary Findings Concerning the Formalin Inactivation of Herpes B, 1112.
with High Complement-Fixing Antigenicity, Production of Foot-and-Mouth Disease, 775.
Vitamin E Administration on Rats Fed Fresh or Autoxidized Beef Tallow, The Effect of, 795.

W

White Muscle Disease (Myopathy) in Lambs and Calves. VII. Etiology and Prophylaxis, 466.
White Muscle Disease, Serum Transaminase in the Blood of Lambs Given Preventive Treatments for, 686.
Wood Preservatives for Swine, Toxicity of, 401.
Wright's Stain, Demonstration of Anaplasma marginale with the Fluorescent Dye, Acridine Orange; Comparisons with the Complement-Fixation Test and, 882.
Wright's Staining Method for Chromosomes and Cell Nuclei, A Rapid, Permanent, 807

HELP US KEEP THE THINGS WORTH KEEPING

A boy keeps days like these all his life. Some day he'll trundle his own sons in a barrow too—remembering the jolly, peaceful man-to-man times spent with his father.

So many precious things like this depend on peace. And *peace* depends upon so many things. For instance: peace costs money.

Money for strength to keep the peace. Money for science and education to help make peace lasting. And money saved by individuals to keep our economy sound.

Every U. S. Savings Bond you buy helps provide money for our country's Peace Power—the power that helps us keep the things worth keeping.

Are you buying as many Bonds as you might?

Photograph by Harold Halma

HELP STRENGTHEN AMERICA'S PEACE POWER

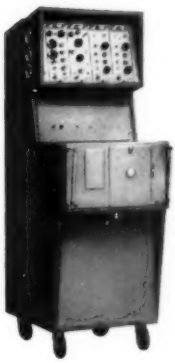
BUY U. S. SAVINGS BONDS

The U.S. Government does not pay for this advertising. The Treasury Department thanks The Advertising Council and this magazine for their patriotic donation.

SANBORN® OPTICAL OSCILLOGRAPHS

NEW DIRECT-READOUT RECORDING SYSTEM

In addition to wide-deflection traces that can overlap, and high frequency response, this new recorder provides the special advantage of direct readout with no chemical developing necessary. Standard galvanometers cover a frequency range of 0 to 500 cycles, providing more than enough response for heart sounds, blood pressures, EEG, ECG, oximetry, dye dilution studies, plethysmograms, temperatures, etc. The Sanborn 350-1700B Heart Sound Preamplifier provides pre-emphasis to extend the heart sound bandwidth to 1000 cycles per second with standard 500 cycle galvanometers. Galvanometers with a frequency range up to 2000 cps are available upon special request for such phenomena as EMG pulses, nerve pulses, etc. The Model 658T uses an 8″ chart, has 9 speeds from 1000 to 2.5 mm/sec, timing lines at 0.1 and 1.0 sec intervals, beam interrupter for trace identification. Monitoring units may be included.



NEW 4-Channel Photographic System
Model 564 Poly-Beam

Efficient system for up to 4 channels, with excellent performance at minimum cost; wide application versatility by using "350" preamplifiers; full-scale (15 cm) recording with clear, reproducible traces. Monitoring units may be mounted on cabinet.

8-Channel Photographic
Model 558M Poly-Beam

Versatile Poly-Beam system uses up to eight "350" preamplifiers; excellent performance, full-scale (15 cm) recording, reproducible traces; monitor units may be included.

2-Channel Photographic Model 62 Twin-Beam

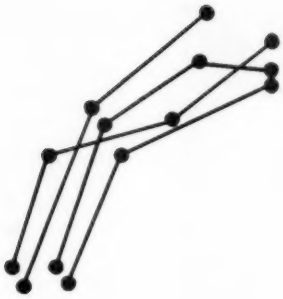
Convenient table-top system provides 6 cm wide recordings plus electrical auscultation; uses Twin-Beam amplifiers (ECG, Phono) or external amplification for additional phenomena; external monitor units may be used.

The extensive and well-known Sanborn line of heated stylus recording systems is available, of course, in addition to the optical systems outlined above. For complete information call the nearest Sanborn Branch Office or Service Agency — or write Manager, Research Instrument Sales:

MEDICAL DIVISION

SANBORN COMPANY

175 Wyman St., Waltham 54, Massachusetts



GREATER PROTECTION

AGAINST CANINE DISTEMPER

• higher levels of protection than ever before reported[1] • 98.1% protection of 8 to 10-week-old dogs—with two 1 cc. doses[2] • pronounced response to booster doses, even in dogs which already have significant antibody levels.

TISSUVAX D-H*

a significant advance in effective immunization of dogs against distemper and hepatitis

References: 1. York, C. J.; Bittle, J. L.; Burch, G. R., and Jones, D. E.: Vet. Med. *55*:30 (April) 1960. 2. York, C. J., and Burch, G. R.: J. Am. Vet. M. A. *138*:298 (March 15) 1961.

*Trademark for CANINE DISTEMPER VACCINE, Modified Live Virus, Chick Tissue Culture Origin, and INFECTIOUS CANINE HEPATITIS VACCINE, Modified Live Virus, Porcine Tissue Culture Origin, Combined, Vacuum Dried.

PITMAN-MOORE COMPANY • ALLIED LABORATORIES
DIVISIONS OF THE DOW CHEMICAL COMPANY
INDIANAPOLIS 6, INDIANA